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Noofeli, Mojtaba (2008) *Genetic analysis and characterisation of the BapC autotransporter of bordetella pertussis*. PhD thesis.

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Genetic analysis and characterisation of the BapC autotransporter of *Bordetella pertussis*



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of Glasgow

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November 2007

**A thesis submitted for the degree of
*Doctor of Philosophy***

Declaration

Unless otherwise stated, this thesis is the original work of the author

M. Noofeli

Dedication

This thesis is dedicated to my wife, Fariba and my daughters, Asoo and Ava, who are always sources of mental support, happiness, love and inspiration for my life and future.

Acknowledgements

I express my best gratitude and most sincere thanks to my supervisors, Dr. Roger Parton and Dr. John Coote for their continued encouragement, supervision, profound enthusiasm, stimulating discussion, patience and reading of, and commenting on this thesis. Their enthusiasm, interest and expertise made this study very enjoyable and educational. I also appreciate and acknowledge the help of Professor Mark Roberts as my assessor throughout my work. I am also grateful to Dr. O. Byron and Dr. R. Aitken for their guidance, helpful advice and collaboration during my work.

My thanks also go to Susan, Irene and all GBRC staff members for their collaboration and help throughout my work.

I must sincerely thank my wife and my children whose support, love and encouragement made this task possible. I am also thankful to my mother-in-law and my mother for their regular calls, mental support and prayers.

I would like to say many thanks to my sponsors, the Ministry of Health and Razi Vaccine Institute for their wonderful support and allowing me to carry out this research work.

Table of contents

DECLARATION.....	I
DEDICATION.....	II
ACKNOWLEDGEMENTS.....	III
TABLE OF CONTENTS.....	IV
LIST OF FIGURES.....	VII
LIST OF TABLES.....	IX
ABBREVIATIONS.....	X
ABSTRACT.....	XIII
1. INTRODUCTION.....	1
1.1 THE GENUS <i>BORDETELLA</i>	1
1.2 SPECIES OF <i>BORDETELLA</i>	2
1.3 PERTUSSIS: THE DISEASE.....	5
1.3.1 Epidemiology of pertussis.....	5
1.3.2 Clinical features.....	6
1.3.3 Pathogenesis.....	8
1.3.4 Diagnosis.....	9
1.3.5 Antimicrobial therapy.....	10
1.3.6 Vaccination and prevention (whole-cell and acellular pertussis vaccines).....	10
1.3.7 Re-emergence of pertussis.....	12
1.4 <i>BORDETELLA PERTUSSIS</i>	13
1.4.1 Virulence factors.....	13
1.4.1.1 Adhesins.....	14
1.4.1.1.1 Fimbriae.....	14
1.4.1.1.2 Filamentous haemagglutinin.....	16
1.4.1.1.3 Pertactin.....	17
1.4.1.1.4 Bordetella resistance to killing protein (BrkA).....	19
1.4.1.1.5 Tracheal colonization factor.....	23
1.4.1.2 Toxins.....	23
1.4.1.2.1 Pertussis toxin (PT).....	24
1.4.1.2.2 Adenylate cyclase toxin (CyaA).....	25
1.4.1.2.3 Dermonecrotic toxin (DNT).....	28
1.4.1.2.4 Tracheal cytotoxin (TCT).....	29
1.4.1.2.5 Lipopolysaccharide.....	30
1.4.2 Other potential virulence factors.....	34
1.4.2.1 Outer-membrane protein P.....	34
1.4.2.2 Outer-membrane protein Q.....	34
1.4.2.3 Bordetella intermediate phase protein.....	35
1.4.2.4 Vir90.....	35
1.4.2.5 Subtilisin-like Ser protease/lipoprotein (SphB1).....	36
1.4.2.6 Virulence-activated gene-8 (vag-8).....	36
1.4.2.7 Pertactin homologous gene (phg).....	37
1.4.3 Regulation of virulence genes.....	37
1.5 OTHER MEMBERS OF <i>B. PERTUSSIS</i> AUTOTRANSPORTER FAMILY.....	41
1.5.1 <i>B. pertussis</i> autotransporter protein-5 (Bap-5) (BapC).....	44
1.6 GENERAL MECHANISMS FOR SECRETION IN GRAM-NEGATIVE BACTERIA.....	48
1.6.1 Signal sequence-independent pathway (Type I):.....	48
1.6.2 General secretory pathway (GSP, Type II):.....	50
1.6.3 Type III secretion pathway (the injectisome):.....	52
1.6.4 Type IV secretion system (T4SS):.....	53
1.6.5 Autotransporters secretion mechanism (Type V secretion).....	55
1.6.5.2 Two-Partner Secretion (TPS) Pathway (Type Vb):.....	61
1.6.5.3 Type Vc (AT-2).....	63
1.6.6 Type VI and VII secretion systems.....	63
1.7 AIMS AND OBJECTIVES.....	64
2. MATERIALS AND METHODS.....	65
2.1 GENERAL BACTERIOLOGICAL PROCEDURES.....	65
2.1.1. Bacterial strains, storage and growth media.....	65
2.1.2. Growth of <i>B. pertussis</i> strains.....	65
2.1.3. Growth of other <i>Bordetella</i> species.....	66

2.1.4. Growth of <i>E. coli</i>	66
2.1.5. Spontaneous antibiotic-resistant strains of <i>B. pertussis</i>	70
2.2 DNA EXTRACTION	70
2.2.1 Isolation of genomic DNA.....	70
2.2.2 Isolation of plasmid DNA	71
2.3 AGAROSE GEL ELECTROPHORESIS	73
2.3.1 Sample preparation	73
2.3.2 Gel preparation and electrophoresis.....	73
2.3.3 Visualisation of DNA	73
2.3.4 Gel extraction procedure	74
2.4 RESTRICTION ENZYME REACTIONS	75
2.5 CLONING PROTOCOL.....	75
2.5.1 DNA preparation	75
2.5.2 Ligation strategies	76
2.6 TRANSFORMATION	76
2.6.1 Preparation of electroporation-competent cells.....	76
2.6.2 Electroporation procedure	77
2.6.3 Preparation of competent cells for heat shock transformation.....	77
2.6.4 Heat shock transformation.....	77
2.7 BACTERIAL CONJUGATION AND GENE REPLACEMENT.....	78
2.7.1 Construction of BapC mutant of BP338.....	78
2.7.2 Construction of BrkA ⁻ , BapC ⁻ double mutant.....	80
2.8 POLYMERASE CHAIN REACTION.....	81
2.8.1 Primers	81
2.8.2 HotStar Taq [™] PCR Method.....	85
2.8.3 Touchdown PCR.....	86
2.8.4 Colony PCR.....	86
2.8.5 Nested PCR.....	87
2.8.6 Multiplex PCR	87
2.8.7 Purification of PCR products	87
2.9 DNA SEQUENCING	88
2.10 SOUTHERN BLOTTING	88
2.10.1 Preparation of digoxigenin-labelled probes	90
2.10.2 Probe hybridisation	91
2.10.3 Colourimetric detection with BCIP/NBT	91
2.10.4 Stripping membrane for reprobing	92
2.11 PROTEIN ANALYSIS.....	93
2.11.1 Fractionation of bacterial cells	93
2.11.2 Expression of recombinant proteins	94
2.11.3 Preparation of bacterial sonicated extract.....	94
2.11.4 Protein estimation.....	95
2.11.5 Sample preparation for electrophoresis	96
2.11.6 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)	96
2.11.7 Western blotting protocol and development of blots.....	97
2.11.8 Protein identification by mass spectrometry.....	98
2.12 IN VITRO TESTS.....	99
2.12.1 Effect of antimicrobial peptides.....	99
2.12.2 Agglutination test.....	100
2.12.3 Serum killing assay	100
2.13 IN VIVO TEST	101
2.13.1 Competition assay for mouse virulence of <i>B. pertussis</i> strains.....	101
2.14 SEQUENCE ANALYSIS TOOLS (BIOINFORMATICS)	103
3. RESULTS.....	105
3.1 CHARACTERISATION OF <i>B. PERTUSSIS</i> STRAINS	105
3.2 CONFIRMATION OF THE BAPC GENOTYPE OF THE <i>B. PERTUSSIS</i> STRAINS	108
3.3 CHARACTERIZATION OF BAPC PROTEIN IN <i>B. PERTUSSIS</i> STRAINS BY IMMUNOBLOTTING	110
3.4 LOCALISATION OF BAPC PROTEIN IN OUTER-MEMBRANE PREPARATIONS OF <i>B. PERTUSSIS</i> STRAINS.....	113
3.5 LOCALISATION OF BAPC IN <i>B. PERTUSSIS</i> STRAINS	117
3.6 SERUM KILLING ASSAYS OF <i>B. PERTUSSIS</i> STRAINS	119
3.7 EXPRESSION AND CHARACTERISATION OF RECOMBINANT BAPC PROTEIN FROM <i>E. COLI</i> M15 PQE60 (ATG1).....	123
3.8 IDENTIFICATION OF BAPC GENE AND BAPC PROTEIN IN RECOMBINANT <i>E. COLI</i>	125
3.9 CHARACTERIZATION OF RECOMBINANT BAPC PROTEIN USING ANTI-HIS-TAGGED ANTIBODY	126
3.10 CHARACTERIZATION OF RECOMBINANT BAPC PROTEIN BY IMMUNOBLOTTING.....	127

3.11	LOCALISATION OF BAPC IN <i>E. COLI</i> STRAINS.....	129
3.12	CHARACTERISATION OF BAPC AS A SERUM-RESISTANCE FACTOR IN <i>E. COLI</i>	132
3.13	IDENTIFICATION OF CLONING AND EXPRESSION VECTORS	135
3.14.1	Construction of <i>E. coli</i> carrying the suicide vector containing <i>bapC::Tet</i>	139
3.14.2	Creation of spontaneous <i>Sm^R</i> derivative of <i>B. pertussis</i> BP338 and <i>brkA</i> mutant.....	148
3.14.3	Conjugation to introduce the constructed plasmid into <i>B. pertussis</i> strains.....	150
3.15	DETERMINING THE SERUM SENSITIVITY OF THE BAPC AND BRKA, BAPC MUTANTS	162
3.16	SUSCEPTIBILITY OF <i>B. PERTUSSIS</i> STRAINS TO ANTIMICROBIAL PEPTIDES	166
3.17	EFFECT OF BAPC PROTEIN ON VIRULENCE OF <i>B. PERTUSSIS</i>	172
3.18	IDENTIFICATION OF THE BAPC LOCUS IN OTHER <i>BORDETELLA</i> SPECIES.....	177
3.19	PREDICTION OF A SIGNAL SEQUENCE IN THE BAPC PROTEIN OF <i>BORDETELLA</i> SPECIES.....	181
3.19.1	Description of the scores	181
3.20	CLONING AND EXPRESSION OF BAPC WITH AND WITHOUT A SIGNAL SEQUENCE	187
3.20.1	Sequencing of cloned <i>bapC</i> and aligning with the <i>bapC</i> in database.....	191
3.20.2	Overexpression of BapC protein in <i>E. coli</i>	191
3.20.3	Identification of BapC protein expressed in <i>E. coli</i>	193
3.21	SEQUENCE DETERMINATION OF BAPC GENE.....	194
3.21.1	Nucleotide sequence analysis of <i>bapC</i> gene region spanning the poly(C) and poly(G) tracts of different <i>B. pertussis</i> strains	196
3.21.2	Amino acid sequence analysis of <i>bapC</i> gene region spanning the poly(C) and poly(G) tracts of different <i>B. pertussis</i> strains	200
4.	DISCUSSION.....	204
4.1	OVERVIEW.....	204
4.2	CONSTRUCTION OF BAPC-DEFECTIVE MUTANTS	206
4.3	ROLE OF BAPC IN SERUM RESISTANCE	209
4.4	EXPRESSION OF RECOMBINANT BAPC	214
4.5	ROLE OF BAPC IN SUSCEPTIBILITY TO ANTIMICROBIAL PEPTIDES (AMPS).....	216
4.6	EFFECT OF BAPC PROTEIN ON VIRULENCE OF <i>B. PERTUSSIS</i> FOR MICE	221
4.7	SEQUENCE OF BAPC GENE IN DIFFERENT <i>B. PERTUSSIS</i> STRAINS AND OTHER <i>BORDETELLA</i> SPECIES.....	223
4.8	COMPARATIVE ANALYSIS OF BAPC WITH BRKA AND PERTACTIN	230
4.8.1	Protein comparison	230
4.8.2	Gene comparison.....	238
4.9	MODELLING/PREDICTION OF BAPC STRUCTURE	241
5.	REFERENCES	247
6.	6. APPENDICES.....	281
6.1	APPENDIX I.....	281
AI.1	Media composition.....	281
AI.2	Molecular biology solutions.....	285
AI.3	Protein analysis solutions	286
AI.4	Southern blot solutions and buffers	289
6.2	APPENDIX II	293
AII.1	Map of the plasmid pBR322 (http://www.fermentas.com/techinfo/nucleicacids/mappbr322.htm)	293
AII.2	Map of pET-33b (+) expression vector (http://www.merckbiosciences.com/docs/docs/PROT/TB147.pdf).....	294
AII.3	Significant features of <i>bapC</i> gene and BapC protein	296
AII.4	<i>brkA</i> gene mutated by transposon 5 insertion and primers (Fernandez and Weiss, 1994).....	299
AII.5	BLAST2 sequence comparison of SapA protein of <i>S. typhimurium</i> with BapC of <i>B. pertussis</i>	300
AII.6	BLAST2 sequence comparison of SapA protein of <i>S. typhimurium</i> with BrkA of <i>B. pertussis</i>	301
AII.7a	Mascot Search Results of full-length BapC protein expressed in <i>E. coli</i>	302
AII.7b	Mascot Search Results of full-length BapC protein expressed in <i>E. coli</i>	303

List of figures

FIGURE 1.1 PHYLOGENETIC RELATIONSHIPS AMONG NINE <i>BORDETELLA</i> SPECIES	4
FIGURE 1.2 VIRULENCE FACTORS OF <i>BORDETELLA PERTUSSIS</i>	14
FIGURE 1.3 STRUCTURAL COMPARISON OF BRKA AND PERTACTIN (PRN).....	20
FIGURE 1.4 THE <i>CYA</i> OPERON	26
FIGURE 1.5 STRUCTURAL ORGANIZATION OF THE <i>CYAA</i> TOXIN.....	27
FIGURE 1.6 SCHEMATIC REPRESENTATION OF THE INNER AND OUTER MEMBRANES OF <i>E. COLI</i> K-12.....	30
FIGURE 1.7 CHEMICAL STRUCTURE OF <i>B. PERTUSSIS</i> LPS (LOS).....	32
FIGURE 1.8 MODEL FOR THE TWO-COMPONENT SIGNAL TRANSDUCTION SYSTEM BY THE BVGAS PROTEINS....	39
FIGURE 1.9 THE <i>BVG</i> LOCUS WITH <i>BVGAS</i> GENES AND THE PROMOTERS P ₁ , P ₂ , P ₃ AND P ₄	40
FIGURE 1.10 BVGAS CONTROLS EXPRESSION OF AT LEAST FOUR CLASSES OF GENES.	41
FIGURE 1.11 STRUCTURAL FEATURES OF SOME AUTOTRANSPORTER PROTEINS IN <i>B. PERTUSSIS</i>	42
FIGURE 1.12 MODEL OF THE TYPE I PROTEIN SECRETION SYSTEM.....	49
FIGURE 1.13 TYPE I SECRETION OF RTX TOXINS	50
FIGURE 1.14 TYPE II SECRETION SYSTEM OF THE PULLULANASE (PULA) IN <i>KLEBSIELLA OXYTOCA</i>	51
FIGURE 1.15 TYPE III SECRETION SYSTEM IN <i>YERSINIA</i>	53
FIGURE 1.16 TYPE IV SECRETION SYSTEM IN <i>H. PYLORI</i>	55
FIGURE 1.17 SCHEMATIC OVERVIEW OF THE TYPE V SECRETION SYSTEMS.....	56
FIGURE 1.18 TYPE VA AUTOTRANSPORTERS.....	57
FIGURE 1.19 MODEL FOR OUTER-MEMBRANE TRANSLOCATION OF PASSENGER DOMAINS	59
FIGURE 1.20 THE TRANSLOCATOR DOMAIN, BECAUSE OF ITS OUTER MEMBRANE PROTEIN CHARACTERISTICS, TARGETS THE OMP85 COMPLEX.....	61
FIGURE 1.21 COMPARISON OF THE AT AND TPS PATHWAYS.	62
FIGURE 1.22 MODEL FOR SECRETION BY THE TPS PATHWAY.	63
FIGURE 2.1 CONSTRUCTION OF <i>BAPC</i> MUTANT OF <i>B. PERTUSSIS</i> BY CONJUGATION OF BP338 STRAIN WITH <i>E.</i> <i>COLI</i> CARRYING SUICIDE VECTOR PSS1129 CONTAINING <i>BAPC::Tet^R</i>	79
FIGURE 3.1 AGAROSE (0.8%) GEL SHOWING THE PCR AMPLIMERS DERIVED FROM THE <i>BAPC</i> GENE IN <i>B.</i> <i>PERTUSSIS</i> STRAINS	109
FIGURE 3.2 IMMUNOBLOTTING WITH ANTI-BAPC SERUM OF WHOLE CELLS OF <i>B. PERTUSSIS</i> TABERMAN STRAINS GROWN IN MODULATING AND NON-MODULATING CONDITIONS.	111
FIGURE 3.3 IMMUNOBLOTTING WITH ANTI-BAPC SERUM OF <i>B. PERTUSSIS</i> TOHAMA STRAINS GROWN IN MODULATING AND NON-MODULATING CONDITIONS	112
FIGURE 3.4 OUTER-MEMBRANE PROTEIN PROFILES OF <i>B. PERTUSSIS</i> STRAINS GROWN IN MODULATING AND NON-MODULATING CONDITIONS	115
FIGURE 3.5 IMMUNOBLOTTING WITH ANTI-BAPC SERUM OF OUTER-MEMBRANE PREPARATIONS OF <i>B. PERTUSSIS</i> STRAINS GROWN IN MODULATING AND NON-MODULATING CONDITIONS.	116
FIGURE 3.6A TIME COURSE OF KILLING OF <i>B. PERTUSSIS</i> STRAINS BY 10% NORMAL HUMAN SERUM (NHS).....	121
FIGURE 3.6B COMPARISON OF SERUM SENSITIVITY OF <i>B. PERTUSSIS</i> STRAINS	122
FIGURE 3.7 PROTEIN PROFILE OF <i>E. COLI</i> STRAINS GROWN IN NON-INDUCED AND INDUCED CONDITIONS WITH IPTG	124
FIGURE 3.8 CALIBRATION OF PROMEGA PROTEIN MOLECULAR WEIGHT MARKERS (SEEBLUE® PLUS2 PRE- STAINED STANDARD)	124
FIGURE 3.9 IMMUNOBLOTTING WITH ANTI-HIS-TAGGED ANTIBODY OF <i>E. COLI</i> STRAINS	127
FIGURE 3.10 IMMUNOBLOTTING WITH UNABSORBED ANTI-BAPC SERUM OF <i>E. COLI</i> STRAINS GROWN IN INDUCED AND NON-INDUCED CONDITIONS.....	128
FIGURE 3.11 IMMUNOBLOTTING WITH ABSORBED ANTI-BAPC SERUM OF <i>E. COLI</i> STRAINS GROWN IN INDUCED AND NON-INDUCED CONDITIONS.....	128
FIGURE 3.12 SERUM SENSITIVITY OF <i>E. COLI</i> M15 PQE60 (ATG1) WITH OR WITHOUT IPTG INDUCTION.....	133
FIGURE 3.13 SERUM SENSITIVITY OF <i>E. COLI</i> M15 PQE60 (NTS) WITH OR WITHOUT IPTG INDUCTION	134
FIGURE 3.14 AGAROSE (0.8%) GEL SHOWING THE PCR PRODUCTS AND EXTRACTED PLASMIDS OF SOME <i>E. COLI</i> STRAINS WITH <i>BAPC</i> SPECIFIC PRIMERS.....	136
FIGURE 3.15 RESTRICTION MAP OF PRTP1 AND PSS1129 (STIBITZ, 1994).....	138
FIGURE 3.16 MAP OF SUICIDE CONSTRUCT pBAPC-Tet [PSS1129 (<i>BAPC::TET</i>)].....	140
FIGURE 3.17 AGAROSE (0.7%) GEL SHOWING PLASMID PSS1129 AND P-B-5 DIGESTED WITH <i>BGL</i> II (A) AND <i>Nco</i> I (B).....	142
FIGURE 3.18 AGAROSE GEL (0.7%) SHOWING THE PCR PRODUCT OF AMPLIFIED TETRACYCLINE GENE OF PBR322 (A) AND pGEMT(Tet) DIGESTED WITH <i>Nco</i> I (B).....	142
FIGURE 3.19 SEQUENCE OF THE TETRACYCLINE RESISTANCE GENE OF PBR322 (ADAPTED FROM WWW.FERMENTAS.COM/TECHINFO/NUCLEICACIDS/MAPPBR322.HTM)	143
FIGURE 3.20 AGAROSE GEL (0.7%) SHOWING AMPLIFIED Tet ^R GENE AND PRODUCTS OF PLASMID P-B-5 AFTER DIGESTION WITH <i>Nco</i> I ± ALKALINE PHOSPHATASE.	144

FIGURE 3.21 AGAROSE GEL (0.7%) SHOWING PCR PRODUCT, USING PRIMERS TCF1 AND TCR1, OF A SELECTED COLONY OF <i>E. COLI</i> CONTAINING PLASMID WITH THE <i>BAPC</i> GENE DISRUPTED BY TET ^R CASSETTE	146
FIGURE 3.22 AGAROSE GEL (0.7%) SHOWING PLASMID pBAPC-Tet (<i>BAPC::TET</i>) FROM <i>E. COLI</i> STRAIN JM109 DIGESTED WITH <i>NcoI</i>	146
FIGURE 3.23 AGAROSE GEL (0.7%) SHOWING THE AMPLIFIED <i>BAPC</i> GENE OF pBAPC-Tet PLASMID FROM <i>E. COLI</i> STRAIN JM109 (FIG. A), AND NESTED PCR OF AMPLIFIED <i>BAPC</i> GENE USING TETRACYCLINE PRIMERS (FIG. B).	147
FIGURE 3.24 AGAROSE GEL (0.7%) SHOWING PLASMID pBAPC-Tet (<i>BAPC::TET^R</i>) FROM <i>E. COLI</i> STRAIN SM10 λ - _{PR} DIGESTED WITH <i>NcoI</i> (A) AND PCR PRODUCT OF AMPLIFIED <i>BAPC</i> GENE DISRUPTED BY TETRACYCLINE RESISTANCE CASSETTE (B).	147
FIGURE 3.25 AGAROSE GEL (0.7%) SHOWING PCR PRODUCT OF STREPTOMYCIN RESISTANT DERIVATIVE OF <i>B. PERTUSSIS</i> <i>BRKA</i> MUTANT (BP2041S) USING <i>BAPC</i> PRIMERS.	149
FIGURE 3.26 OUTER-MEMBRANE PROTEIN PROFILES OF <i>B. PERTUSSIS</i> STRAINS	149
FIGURE 3.27 STRATEGY ADOPTED FOR REPLACEMENT OF <i>BAPC</i> GENE WITH <i>BAPC</i> WITH INSERTED TETRACYCLINE RESISTANCE CASSETTE.....	151
FIGURE 3.28 AMPLIFIED <i>BAPC</i> GENE OF BP338 (<i>BAPC⁻</i>) AND BP338 (<i>BRKA⁻, BAPC⁻</i>) MUTANT STRAINS DISRUPTED BY TETRACYCLINE CASSETTE (FIG. A) AND TETRACYCLINE GENE INSERTED IN <i>BAPC</i> GENE AMPLIFIED BY NESTED PCR (FIG. B).	153
FIGURE 3.29 AMPLIFIED <i>BAPC</i> GENE OF BP338S AND BP2041S STRAINS USING <i>BAPC</i> PRIMERS.....	153
FIGURE 3.30 AMPLIFIED <i>BAPC</i> GENE IN A SELECTED COLONY SHOWING A NON-DOUBLE CHROMOSOMAL CROSS-OVER	154
FIGURE 3.31 5'-TERMINUS (B) AND 3'-TERMINUS (A) AMPLIFICATION OF THE MUTATED <i>BAPC</i> GENE IN CONSTRUCTED MUTANTS USING <i>BAPC</i> FORWARD AND TET REVERSE PRIMERS (B); AND TET FORWARD AND <i>BAPC</i> REVERSE PRIMERS (A), RESPECTIVELY.	154
FIGURE 3.32 DIGESTION OF GENOMIC DNA OF <i>B. PERTUSSIS</i> STRAINS WITH <i>XhoI</i> ENZYME	157
FIGURE 3.33 SOUTHERN BLOTTING OF DIGESTED GENOMIC DNA OF <i>B. PERTUSSIS</i> STRAINS WITH A <i>BAPC</i> -SPECIFIC PROBE	158
FIGURE 3.34 AGAROSE GEL 0.7% SHOWING AMPLIFIED WILD-TYPE <i>BAPC</i> AND <i>BRKA</i> GENES BY MULTIPLEX PCR USING <i>BAPC</i> AND <i>BRKA</i> PRIMERS.....	159
FIGURE 3.35 DNA GENOMIC DIGESTION OF <i>B. PERTUSSIS</i> STRAINS WITH <i>Clai</i> , <i>EcoRI</i> AND <i>EcoRV</i> ENZYMES.....	160
FIGURE 3.36 SOUTHERN BLOTTING OF DIGESTED GENOMIC DNA OF <i>B. PERTUSSIS</i> STRAINS WITH A <i>BRKA</i> -SPECIFIC PROBE	161
FIGURE 3.37 COMPARISON OF SERUM KILLING OF <i>B. PERTUSSIS</i> STRAINS (1/40 HUMAN SERUM)	164
FIGURE 3.38 COMPARISON OF SERUM KILLING OF <i>B. PERTUSSIS</i> STRAINS (1/80 HUMAN SERUM)	164
FIGURE 3.39 COMPARISON OF SERUM KILLING OF <i>B. PERTUSSIS</i> STRAINS GROWN IN MODULATING AND NON-MODULATING CONDITIONS (1/40 HUMAN SERUM)	165
FIGURE 3.40 COMPARISON OF SERUM KILLING OF <i>B. PERTUSSIS</i> STRAINS GROWN IN MODULATING AND NON-MODULATING CONDITIONS (1/80 HUMAN SERUM)	165
FIGURE 3.41 SUSCEPTIBILITY OF <i>B. PERTUSSIS</i> STRAINS TO THE ANTIMICROBIAL PEPTIDE CECROPIN P1 (0.25 μ M)	168
FIGURE 3.42 SUSCEPTIBILITY OF <i>B. PERTUSSIS</i> STRAINS TO THE ANTIMICROBIAL PEPTIDE PROTAMINE (2.4 μ M)	169
FIGURE 3.43 SUSCEPTIBILITY OF <i>B. PERTUSSIS</i> STRAINS TO THE ANTIMICROBIAL PEPTIDE MASTOPARAN (0.48 μ M)	170
FIGURE 3.44 EFFECT OF GROWTH OF TWO STRAINS INPUT MIXTURE ON BG AGAR.....	175
FIGURE 3.46 AGAROSE GEL (0.7%) SHOWING THE PCR PRODUCTS OF <i>BORDETELLA</i> GENOMIC DNAs USING FORWARD AND REVERSE <i>BAPC</i> PRIMERS.....	177
FIGURE 3.47 <i>BORDETELLA</i> GENOMIC DNAs DIGESTED WITH <i>XhoI</i> (A), <i>BglII</i> (B) AND <i>NdeI</i> (C) ENZYMES AND ANALYSED BY GEL ELECTROPHORESIS (0.7% AGAROSE)	179
FIGURE 3.48 SOUTHERN BLOT ANALYSIS OF <i>BORDETELLA</i> GENOMIC DNAs, DIGESTED WITH <i>XhoI</i> (A), <i>BglII</i> (B), AND <i>NdeI</i> (C), WITH <i>BAPC</i> PROBE.....	180
FIGURE 3.49 SIGNALP-NN RESULT OF <i>BAPC</i> IN <i>BORDETELLA PERTUSSIS</i>	184
FIGURE 3.50 SIGNALP-NN RESULT OF <i>BAPC</i> IN <i>BORDETELLA PARAPERTUSSIS</i>	185
FIGURE 3.51 SIGNALP-NN RESULT OF <i>BAPC</i> IN <i>BORDETELLA BRONCHISEPTICA</i>	186
FIGURE 3.52 AGAROSE GEL (0.7%) SHOWING PURIFIED DIGESTED pET33B VECTOR.....	189
FIGURE 3.53 AGAROSE GEL (0.7%) SHOWING DIGESTED AND PURIFIED PCR PRODUCTS OF <i>BAPC</i> GENE WITH SIGNAL SEQUENCE (A) AND DIGESTED PURIFIED NESTED GRADIENT PCR PRODUCTS OF <i>BAPC</i> GENE WITHOUT SIGNAL SEQUENCE (B).	189
FIGURE 3.54 AGAROSE GEL (0.7%) SHOWING PCR PRODUCTS OF SELECTED CLONES USING <i>BAPC</i> PRIMERS AFTER PLASMID PURIFICATION	190
FIGURE 3.55 AGAROSE GEL (0.7%) SHOWING PURIFIED PLASMID FROM SELECTED CLONES DIGESTED WITH <i>NHEI/BAMHI</i> AND <i>NDEI/BAMHI</i> ENZYMES.....	190
FIGURE 3.56 PROTEIN PROFILE SHOWING THE WHOLE-CELL FRACTIONS OF <i>E. COLI</i> BL21 (DE3) pLYsS CONTAINING pET33B (+) VECTOR EXPRESSING <i>BAPC</i> PROTEIN WITH OR WITHOUT THE SIGNAL SEQUENCE	192

FIGURE 3.57 AGAROSE GEL (0.7%) SHOWING THE AMPLIFIED FRAGMENT OF <i>BAPC</i> GENE ENCOMPASSING THE POLY(C) REGION, USING PRIMERS MNF1, MNR1	195
FIGURE 3.58 AGAROSE GEL (0.7%) SHOWING AMPLIFIED FRAGMENT OF <i>BAPC</i> GENE ENCOMPASSING THE POLY(G) REGION, USING PRIMERS MNF2, MNR2	195
FIGURE 3.59 CLUSTAL W (1.83) MULTIPLE SEQUENCE ALIGNMENT OF 5'-END OF <i>BAPC</i> GENE ENCOMPASSING POLY(C) SITES	197
FIGURE 3.60 CLUSTAL W (1.83) MULTIPLE SEQUENCE ALIGNMENT OF 5'-END OF <i>BAPC</i> GENE ENCOMPASSING POLY(G) SITES	198
FIGURE 3.61 CLUSTAL W (1.83) MULTIPLE SEQUENCE ALIGNMENT OF AMINO ACIDS AT N-TERMINUS OF <i>BAPC</i> , CORRESPONDING TO REGION CONTAINING THE POLY(C) SEQUENCES	201
FIGURE 3.62 CLUSTAL W (1.83) MULTIPLE SEQUENCE ALIGNMENT OF AMINO ACIDS AT N-TERMINUS OF <i>BAPC</i> , CORRESPONDING TO REGION CONTAINING THE POLY(G) SEQUENCES	201
FIGURE 4.1 ALIGNMENT OF PROTEIN SEQUENCES OF BRKA, <i>BAPC</i> AND PERTACTIN	232
FIGURE 4.2 PREDICTION OF THE SECONDARY STRUCTURE OF <i>BAPC</i> BY PROTEUS TOOL	243
FIGURE 4.3 MODEL OF THE TERTIARY STRUCTURE OF THE PASSENGER DOMAIN OF THE <i>BAPC</i> PROTEIN OBTAINED USING THE SWISS-MODEL PROGRAMME	245
FIGURE 4.4 TERTIARY STRUCTURE OF PERTACTIN (DAUTIN AND BERNSTEIN, 2007)	245

List of Tables

Table 1.1 <i>Bordetella</i> autotransporter proteins	43
Table 2.1 <i>E. coli</i> strains used in this study	67
Table 2.2 <i>Bordetella</i> strains used in this study	68
Table 2.3 Antibiotics used in this study	69
Table 2.4 Plasmids used in this study	72
Table 2.5 Primers used in this study	82
Table 3.1 Antibiotic sensitivities and other features of <i>B. pertussis</i> strains available at the start of this study	107
Table 3.2 Agglutination of <i>B. pertussis</i> strains grown in modulating and non-modulating conditions	118
Table 3.3 Slide agglutination test with different <i>E. coli</i> expression strains in induced and non-induced conditions with IPTG	131
Table 3.4 Number of C and G residues in the <i>bapC</i> poly(C) and poly(G) tracts in <i>B. pertussis</i> strains	199

Abbreviations

ACT	Adenylate cyclase toxin
ACV	Acellular vaccine
ACS	American Chemical Society
ADP	Adenosine diphosphate
Amp	Ampicillin
AMPs	Antimicrobial peptides
Ap (aP)	Acellular pertussis
APS	Ammonium persulphate
AT(s)	Autotransporter(s)
ATP	Adenosine triphosphate
BapC	<i>Bordetella</i> autotransporter protein C
BipA	<i>Bordetella</i> intermediate phase protein A
BLAST	Basic local alignment tool
BG	Bordet-Gengou
BrkA	<i>Bordetella</i> resistance to killing protein A
BrkB	<i>Bordetella</i> resistance to killing protein B
BSA	Bovine serum albumin
Bvg	<i>Bordetella</i> virulence gene
CAA	Casamino acids
CaM	Calmodulin
cAMP	Adenosine 3', 5'-cyclic monophosphate
CDC	Centers for Disease Control and Prevention
cfu	Colony forming unit
CGH	Comparative genome hybridization
CHO	Chinese hamster ovary
CL	Cyclodextrin liquid
CRD	Carbohydrate recognition domain
CR3	Complement receptor type III
CyaA	Adenylate cyclase toxin
DFA	Direct fluorescent antibody
dH ₂ O	Distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNT	Dermonecrotic toxin
dNTP	Deoxynucleoside triphosphate
dsDNA	Double-stranded DNA
DTaP	Diphtheria, tetanus, acellular pertussis
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FHA	Filamentous haemagglutinin
Fim	Fimbriae
g/mg/μg/ng	Gram/milligram/microgram/nanogram
GalNAc	N-acetyl galactosamine
gfp	Green fluorescent protein
GlcA	Glucuronic acid
GlcN	Glucosamine
GSP	General secretory pathway
GTP	Guanosine triphosphate
h	Hour
HIV	Human immunodeficiency virus
Hib	<i>Haemophilus influenzae</i> type b

HPD	Histidine phosphotransfer domain
HTH	Helix-turn-helix
ICAM-1	Intercellular adhesion molecule 1
IM	Inner membrane
IPTG	Isopropyl β -D-thiogalactopyranoside
IPV	Inactivated poliovirus vaccine
IU	International units
KDO	3-deoxy-d-manno-oct-2-ulosonic acid
Km	Kanamycin
l/ml/ μ l	Litre/millilitre/microlitre
LB	Luria Bertani
LC	LightCycler
LD ₅₀	50% lethal dose
LOS	Lipo-oligosaccharide
LPS	Lipopolysaccharide
M/mM/ μ M	Molar/millimolar/micromolar
MBL	Mannan Binding Lectin
MFP	Membrane fusion protein
MgSO ₄	Magnesium sulphate
min	Minute
MLEE	Multilocus enzyme electrophoresis
MLST	Multilocus sequence typing
MDO	Membrane-derived oligosaccharide
mRNA	Messenger RNA
MTB	Main terminal branch
NaCl	Sodium chloride
NAD	Nicotinamide adenine dinucleotide
Nal	Nalidixic acid
NIBSC	National Institute for Biological Standards and Control
NO	Nitric oxide
OD _{xxxnm}	Optical density at xxx _{nm} wavelength
OMP	Outer membrane protein
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PCR/LDR	Polymerase chain reaction/ligase detection reaction
PEtN	Phosphoethanolamine
PFGE	Pulsed field gel electrophoresis
<i>phg</i>	Pertactin homologous gene
PICU	Paediatric intensive care unit
PMF	Proton motive force
POTRA	Polypeptide-transport-associated
PPi	Pyrophosphate
PPEtn	Phosphoethanolamine
PRN (Prn)	Pertactin
psi	pound-force per square inch
PT	Pertussis toxin
<i>ptx</i>	Pertussis toxin gene
RGD	Arg-Gly-Asp
RNA	Ribonucleic acid
rpm	Revolutions per minute
rRNA	Ribosomal RNA
RT-PCR	Reverse transcriptase PCR

RTX	Repeats in toxin
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sec	Seconds
SNP	Single nucleotide polymorphism
SOC	Super optimal catabolite
Sm	Streptomycin
SS	Stainer-Scholte
TBE	Tris-Borate-EDTA
TCF (Tcf)	tracheal colonisation factor
TCT	Tracheal cytotoxin
TE	10mM Tris, 1mM EDTA
Tet	Tetracycline
Tm	Melting temperature
Tn5	Transposon 5
TPS	Two-partner secretion system
U	Units
<i>vag</i>	virulence activated gene
Vag8	Virulence-activated gene 8 protein
VLA-5	Very late antigen-5
<i>vrg</i>	Virulence-repressed gene
WCV	Whole-cell vaccine
WHO	World Health Organization

Abstract

The autotransporters are a family of extracellular proteins, found in various Gram-negative bacteria, that have many different functions but appear to have a similar mechanism of export. In *B. pertussis*, the virulence-regulated proteins Pertactin, BrkA, Tcf, and Vag8 have structural homology at their C-termini (30-kDa) and the N-terminal of the mature proteins share structural characteristics such as RGD and SGXG motifs. Recently, another member of the *B. pertussis* autotransporter family, Bap-5 (Blackburn, 2000) (GenBank accession number AF081494) or BapC (GenBank accession number AJ277634.1) was identified. Subsequent work by Bokhari (2002) suggested that BapC, like BrkA (Fernandez and Weiss, 1994), could function as a serum-resistance factor and was important in colonization of the mouse respiratory tract. However, the relative contributions of these two components to serum resistance and mouse virulence were difficult to estimate because the studies had been done with *brkA* and *bapC* mutants of different *B. pertussis* strains.

The first part of the current project involved the creation of *bapC* and *brkA*, *bapC* mutants in the same genetic background as the *brkA* mutant, namely *B. pertussis* strain BP338, an antibiotic-resistant derivative of strain Tohama. When these strains were compared to the wild-type strains available, *B. pertussis* Tohama (wild-type, W/T), Taberman (W/T) and BP338, they were shown to be less resistant to killing by normal human serum. The *brkA*, *bapC* double mutant of BP338 was more serum sensitive than the *bapC* or *brkA* single mutants but not as sensitive as a *bvg* mutant BP347, also a derivative of strain BP338, which lacks expression of almost all known virulence factors. These findings supported the suggestion that BapC, like BrkA, is a serum-resistance factor but also indicated that other *bvg*-regulated factor(s) may be able to confer some serum resistance on *B. pertussis*. In addition, an *E. coli* strain previously constructed by Blackburn (2000) expressing a large part of the BapC protein was shown to be more serum-resistant than control strains.

It had been reported previously that the *brkA* mutant of *B. pertussis* BP338 was not only more sensitive to serum, but also had altered sensitivity to certain antimicrobial peptides (Fernandez and Weiss, 1996). In the present study, the *bapC* and *brkA* mutants were found to be more sensitive to cecropin P1 than the parent strain, and the double mutant and the *bvg* mutant were even more sensitive. In contrast, the *bapC* and *brkA* mutants were more resistant to protamine whereas the double mutant and *bvg* mutant were even more resistant.

Competition assays, by intranasal infection of mice, were done to compare the virulence of the mutant strains with that of the parent strain BP338. The order of virulence determined by these assays was: BP338 (W/T) > BP338 (BrkA⁻) > BP338 (BapC⁻) > BP338 (BrkA⁻, BapC⁻) > BP347 (Bvg⁻), which is approximately the same as the order of resistance to serum killing: BP338 (W/T) > BP338 (BapC⁻) ≥ BP338 (BrkA⁻) > BP338 (BrkA⁻, BapC⁻) > BP347 (Bvg⁻) and resistance to antimicrobial peptide (cecropin P1): BP338 > BP338 (BapC⁻) = BP338 (BrkA⁻) > BP338 (BrkA⁻, BapC⁻) > BP347 (Bvg⁻). These findings suggest that BapC, like BrkA, is an important virulence determinant of *B. pertussis* and also that resistance to serum killing and possibly to certain antimicrobial peptides may have important roles in virulence.

During the course of the current study, the genome sequences of *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* were published (Parkhill *et al.*, 2003) and it became evident that the *bap5/bapC* sequences identified previously were not, in fact, the whole gene. It also became evident that *bapC* in the Tohama strain of *B. pertussis* used to obtain the genome sequence was actually a pseudogene, due to a frameshift in homopolymeric tract of C residues at the 3' end of the gene. This information clearly conflicted with the evidence from the current and previous studies from our laboratory that BapC was expressed and conferred serum resistance and virulence properties on the producing strains. Nucleotide and amino acid analyses of the *bapC* region spanning the poly(C) and poly(G) tracts of a number of *B. pertussis* strains in this study showed some minor

nucleotide and amino acid polymorphisms in some strains but it appeared that all had an ORF that would be able to produce some form of BapC.

DNA hybridisation of a *bapC* probe to the genomes of other *Bordetella* species showed the presence of the *bapC* gene in *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* but there was no evidence of the *bapC* locus or orthologous gene in *B. avium*, *B. hinzii*, *B. holmesii* or *B. trematum*.

Prediction of the signal sequence of BapC in *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* was performed according to the amino acid sequences available in the database (GenBank) and showed a maximum cleavage site probability between amino acids 38 and 39. Other comparative analysis of proteins and genes between BapC, BrkA and pertactin clearly revealed the homology in their C-termini (β -domains), as would be expected for autotransporter proteins. However, apart from some common motifs (RGD, SGXG), no clear homology could be seen in their passenger domains (α -domains) to suggest common functions.

The entire *bapC* gene was cloned from BP338 and expressed in *E. coli* and a band of ~90 kDa was obtained by SDS-PAGE. The predicted size of BapC is 104 kDa, comprising a 74 kDa α -domain and 30 kDa β -domain. Peptide fingerprinting and mass spectrometry analysis showed that this band corresponded, with a high peptide coverage, to the BapC protein published in the database (GenBank), without the frameshift, and confirmed that BapC would be expressed in *B. pertussis* strain BP338. This recombinant BapC, free from other *B. pertussis* components, will be very useful for further studies, for example in determining the mechanism of serum resistance and its potential as a protective antigen for inclusion in any new generation of acellular pertussis vaccines.

1. Introduction

Bordetella pertussis, the causative agent of pertussis or whooping cough, was first isolated and implicated as the cause of the disease in 1906 (Bordet and Gengou, 1906). Whooping cough is presently one of the ten most common causes of death from infectious disease. It was reported by the World Health Organization (WHO) to cause 50 million cases and 294,000 deaths worldwide in 2002, mainly among unvaccinated individuals in Third World countries. In the USA, where there is a high vaccine uptake, the Centers for Disease Control and Prevention recorded 18,957 reported cases in 2004. In the UK, 1054 cases were reported in 2002, despite a vaccine uptake of 95% (WHO, Statistics, 2006). The introduction of a pre-school booster vaccination is predicted to reduce the number of hospitalizations by approximately 1400 and pertussis cases in the UK by up to 28000 over a 5-year period (Stevenson *et al.*, 2002).

1.1 The genus *Bordetella*

The genus *Bordetella* is comprised of minute (0.2-0.5µm x 0.5-2.0 µm), Gram- negative, catalase-positive, non-acid fast, non-sporing, aerobic (with the exception of *B. petrii*) coccobacilli. They do not ferment carbohydrates. Members of the genus *Bordetella* (except for *B. petrii*) appear to be obligatory parasites, mostly inhabiting the surface of the respiratory tract of man and other warm-blooded animals, including birds. According to Lapin (1943), the small Gram-negative coccobacillus, now known as *B. pertussis*, was first clearly described in 1906, by Bordet and Gengou, in the sputum of an infant with whooping cough, although other investigators had noted similar organisms previously. By 1906, Bordet and Gengou had developed a suitable medium for cultivation of the organism and were then able to establish its morphology, cultural characteristics, virulence and antigenicity. The G+C content of the genus is in the range of 61-68% and the optimum growth temperature is 35-37°C (Parton, 1998).

By comparative 16S rRNA sequence analysis, amplified ribosomal DNA restriction analysis and DNA-RNA hybridization studies and the high G+C content in their DNA, it has been suggested that the *Bordetella* species are closely related to *Achromobacter* species. *Achromobacter* is a genus of Gram-negative, aerobic, motile bacteria that occurs in water and soil. Some are common inhabitants of the intestinal tract of vertebrates and they occasionally cause opportunistic infections in humans. Evidence from 16S rRNA sequence

analysis has also confirmed a common phylogeny with *Alcaligenes* and it may be difficult to differentiate these genera on the basis of phenotypic criteria (Parton, 1998).

1.2 Species of *Bordetella*

DNA-DNA hybridization analyses, T_m values of rRNA-DNA hybrids, mean G + C contents of genomic DNA, similarity in 23S rRNA gene sequences, and whole genomic sequencing have suggested a very close relationship between *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* (van der Zee *et al.*, 1997). *B. pertussis* strains, which are restricted to humans and the human respiratory tract, show little genetic variation, indicating that the species derived from a common ancestor in the recent past, perhaps only a few thousand years ago. *B. parapertussis* infects both humans and sheep; in human infants it may cause whooping cough. Phylogenetic analysis has shown that *B. parapertussis* strains isolated from humans are distinct from those isolated from sheep. It has been suggested that the two types evolved independently from a common ancestor (*B. bronchiseptica*), and there is little or no transmission between the two reservoirs (sheep and human). Furthermore, isolates from sheep are genetically diverse, whereas those isolated from humans are more uniform. In contrast, *B. bronchiseptica* has a broad host range, causing chronic and often asymptomatic respiratory tract infections in a wide range of animals, and only occasionally causes infection in humans (Goodnow, 1980), or opportunistic infection in the immunocompromised (Kerr and Matthews, 2000).

B. pertussis, *B. parapertussis* and *B. bronchiseptica* were the first species assigned to the genus *Bordetella*. In recent years, six new species have been added to the genus. *B. avium* was first proposed as a species by Kersters *et al.* (1984) to include agents of respiratory disease of turkey poults and other birds. *B. hinzii* is the name given to a *B. avium*-like group of organisms found mainly in the respiratory tracts of chickens and turkeys but human isolates have been reported (Vandamme *et al.*, 1995). It was reported that *B. hinzii* might cause disease in humans under certain conditions e.g. in HIV and cystic fibrosis patients (Funke *et al.*, 1996; Kattar *et al.*, 2000). *B. holmesii* was originally isolated from human blood cultures (Weyant *et al.*, 1995) but also from sputum (Tang *et al.*, 1998) and nasopharyngeal specimens from patients with pertussis-like symptoms (Mazengia *et al.*, 2000).

A recently named species, *B. trematum*, contains isolates from human wound and ear infections which were described originally as atypical bordetella or unidentified

(Vandamme *et al.*, 1996). The pathogenic potential of *B. hinzii*, *B. holmesii* and *B. trematum* is unclear, although *B. hinzii* has been identified as the cause of a fatal septicemia (Kattar *et al.*, 2000). Like *B. bronchiseptica*, they appear to be opportunistic in humans although it has been reported that there are no hosts other than humans for *B. holmesii* and *B. trematum*.

More recently, *B. petrii* has been described (von Wintzingerode *et al.*, 2001). This is the first member of this genus to be isolated from the environment and may therefore be very closely related to a common ancestor of the pathogenic *Bordetella*. Only one strain has been isolated, from an anaerobic, dechlorinating bioreactor culture enriched from river sediment. This strain, Se-1111R (T), is able to anaerobically reduce selenate to elemental selenium. *B. petrii* is also the first member of this genus capable of anaerobic growth (von Wintzingerode *et al.*, 2001).

It has been confirmed by using multilocus enzyme electrophoresis (MLEE) and microarray-based comparative genome hybridization (CGH) that there is a close genetic relationship between all known bordetellae, with the *B. petrii* facultative anaerobe as the proposed environmental progenitor of pathogenic bordetellae (Cummings *et al.*, 2004; Mattoo and Cherry, 2005) (Fig. 1.1). Moreover, *B. bronchiseptica* seems to be the evolutionary progenitor of *B. pertussis*, *B. parapertussis*_{hu}, and *B. parapertussis*_{ov}; as such, these species have been reclassified as subspecies of the “*B. bronchiseptica* cluster” (Mattoo and Cherry, 2005).

Very recently, a new *Bordetella* species, *B. ansorpii*, was isolated from the purulent exudate of an epidermic cyst (Ko *et al.*, 2005), and the first UK clinical isolate was found in an immunocompromised patient (Fry *et al.*, 2007). It is a Gram-negative bacillus and grows on both blood agar and MacConkey agar. The organism is motile on LB swarming agar (0.8% NaCL, 0.4% agar [wt/vol]), which distinguishes it from *B. holmesii*. Sequence analysis of the 16S rRNA gene, and comparative analysis of two protein coding genes (*risA* and *ompA*) have revealed that this bacterium belongs to the genus *Bordetella* but is distinct from previously described *Bordetella* species (Ko *et al.*, 2005). Cellular fatty acid analysis and biochemical tests have also confirmed that this bacterium is separate from other *Bordetella* species. The G+C content of *B. ansorpii* is 63.8 mol%, and compares with that of other *Bordetella* species which range from 60 to 69 mol%.

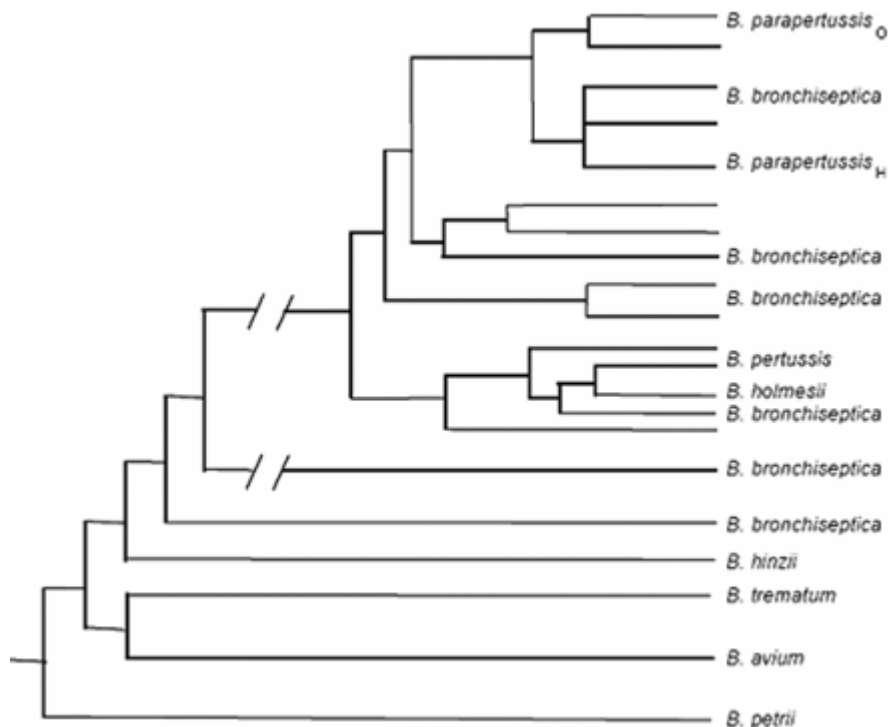


Figure 1.1 Phylogenetic relationships among nine *Bordetella* species based on multilocus enzyme electrophoresis (MLEE), IS element, and sequence analysis. These species appear to have derived from a common *B. petrii* ancestor (Mattoo and Cherry, 2005).

Various studies on the relatedness of the above strains have suggested that *B. pertussis*, *B. parapertussis*, *B. bronchiseptica* are in fact subtypes of a single genomic species. *B. avium*, *B. holmesii*, *B. hinzii*, *B. trematum* and perhaps *B. petrii* and *B. ansorpii* each in contrast form true genomic species (Vandamme *et al.*, 1995, 1996; Weyant *et al.*, 1995; Ko *et al.*, 2005). Evolutionary history of *B. pertussis*, *B. parapertussis*, *B. bronchiseptica* and their close phylogenetic relationship with *Alcaligenes* and other bacteria widespread in the environment suggests that the ancestral bordetellae were free-living and evolved to infect warm-blooded animals (Roberts and Parton, 2001). These suggestions have been supported by the more recent isolation and characterization of *B. petrii* (Matto and Cherry, 2005). Genotyping studies, by multi-locus enzyme electrophoresis and the distribution of IS elements, have suggested that *B. pertussis* and *B. parapertussis* evolved at different times from distinct clones of *B. bronchiseptica* (van der Zee *et al.*, 1996). In addition, the sequence comparison and genome sizes of *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* also support the hypothesis that *B. pertussis* and *B. parapertussis* recently and independently evolved from *B. bronchiseptica*-like ancestors (Parkhill *et al.*, 2003). Moreover, other evidence from DNA fingerprinting by PFGE (Pulsed-Field Gel

Electrophoresis) and arbitrarily-primed PCR (Yuk *et al.*, 1998) shows that *B. pertussis* strains and human strains of *B. parapertussis* are both still very homogeneous clonal populations, supporting the suggestion of their relatively recent adaptation to humans (Parkhill *et al.*, 2003).

1.3 Pertussis: The disease

1.3.1 Epidemiology of pertussis

Bordetella pertussis is the causative agent of pertussis or whooping cough, a serious and highly contagious disease of the respiratory tract, presently responsible for one of the ten most common causes of death from infectious diseases worldwide (Preston and Kerr, 2001). It has historically been reported that the attack rate in females was higher than in males (Cherry, 1984). Despite a high vaccine uptake, resurgences of this disease have been observed in several developed countries in the last two decades (Kerr and Matthews, 2000). Throughout the previous century infants and young children have remained most susceptible to pertussis-related morbidity and mortality. Infants younger than 6 months who have not received three doses of the diphtheria-tetanus-pertussis vaccine and under-vaccinated preschool children are at highest risk for pertussis-associated complications. In the United States, in spite of a stable or increasing vaccination rate since 1962, pertussis infection rates have been rising since the early 1980s. In 1993, the pertussis infection rate increased by 82% over reported cases during the previous year and it was the highest incidence rate of pertussis since 1967 (Black, 1997).

In the United States, pertussis was reported as the most common vaccine-preventable disease among children younger than 5 years old in 1993. Although, widespread immunization has reduced the potential for individuals to acquire exposure-induced immunity, the primary factor contributing to the resurgence of pertussis in the US was the growth of a susceptible adult population (Black, 1997). In the UK, following an increased incidence in the late 1970s and early 1980s due to a reduction in vaccine uptake, infection rates fell again once vaccination uptake had resumed and remained low in the 1990s [2390 cases were reported in 1996, despite a vaccine uptake of 95% (Miller, 1999)]. In most countries the disease is endemic, with regular epidemic cycles occurring every 3-5 years, both at a low level in immunized populations and at a high level in populations without vaccination (Kerr and Matthews, 2000). This traditional cycle pattern happens because it

takes a few years to develop a significant number of susceptibles in the population so that widespread transmission can occur.

The average yearly incidence of reported or observed pertussis in the United States in the prevaccine period was 157 cases per 100,000 populations (Cherry, 1984). The average yearly incidence of reported pertussis in the prevaccine era in England and Wales was 230 per 100,000 populations. However, it has been estimated that only 18% of cases were reported in the United States, giving an actual rate in the range of 872 cases per 100,000 populations. Pertussis was an ever-present disease in the prevaccine period, and the epidemic cycles were every 2 to 5 years (on average about every 3 years) (Fine and Clarkson, 1982). The average interval between epidemic peaks in the prevaccine era in the USA (1922 to 1942) was similar to that in the early vaccine era (1962 to 1982) and the present vaccine era (1983 to 2004) (Cherry, 2003).

It has been suggested that adults are now the majority of individuals that become infected with pertussis. Adults are often found to be the first case in a household with multiple pertussis cases. The importance of pertussis has been confirmed as the cause of persistent cough among teenagers and adults (Mattoo and Cherry, 2005). Adolescents and adults have thus become an important source of transmission of pertussis to other household members particularly infants and young children who are not adequately immunized since the diagnosis of pertussis often goes unrecognized in these older-age groups and treatment is delayed or administered only partially (Black, 1997).

Pertussis is highly contagious and secondary attack rates may exceed 80% among susceptible household contacts. Transmission occurs by direct contact with respiratory secretions or via aerosol droplets from the respiratory tract of infected people. The organism is not thought to survive in the environment for a prolonged period and so transmission must occur from one individual to another (Black, 1997; Cherry, 1999). It is generally accepted that prolonged human carriage does not occur.

1.3.2 Clinical features

The clinical features of pertussis tend to vary with age, general health and immune status. For example adults and older children often show mild or atypical symptoms (Roberts and Parton, 2001). The incubation period of pertussis is commonly 7 to 10 days, with a range of

4 to 21 days, and rarely may be as long as 42 days. The clinical course of the illness is divided into three stages.

The first stage, the catarrhal stage, is characterized by the onset of coryza (runny nose), sneezing, low-grade fever, and a mild, occasional cough, and is similar to the common cold. The patient is most infectious during the initial stage of the disease before it is clearly distinguishable from a common cold. The cough gradually becomes more severe and, after 1-2 weeks, the second or paroxysmal stage begins.

It is during the paroxysmal stage that the diagnosis of pertussis is usually suspected. Characteristically, the patient has bursts, or paroxysms of numerous, rapid coughs, apparently due to difficulty in expelling thick mucus from the tracheobronchial tree. At the end of the paroxysm, a long inspiratory effort is usually accompanied by a characteristic high-pitched whoop. During such an attack, the patient may become cyanotic (turn blue). Children and young infants, especially, appear very ill and distressed. Vomiting and exhaustion commonly follow the episode. The patient usually appears normal between attacks.

Paroxysmal attacks occur more frequently at night, with an average of 15 attacks per 24 hours. During the first 1 or 2 weeks of this stage the attacks increase in frequency, then remain at the same level for 2 to 3 weeks, and then gradually decrease. The paroxysmal stage usually lasts 1 to 6 weeks, but may persist for up to 10 weeks. Infants under 6 months of age may not have the strength to have a whoop, but they do have paroxysms of coughing.

In the convalescent stage, recovery is gradual. The cough becomes less paroxysmal and disappears over 2 to 3 weeks. However, paroxysms often persist with subsequent respiratory infections, many months after the commencement of pertussis.

During the course of pertussis, fever is generally minimal. Older persons (i.e., adolescents and adults), and those incompletely protected by the vaccine, typically have milder disease. Pertussis in these persons may present as a continual (>7 days) cough, and may be difficult to differentiate from other upper respiratory infections. Up to 7% of cough illnesses per year in adults is estimated to be because of pertussis infection. Although the disease may be milder in older persons, these infected persons may transmit the disease to other vulnerable persons, including unimmunized or under-immunized infants. Secondary infections, such as otitis media and pneumonia, are relatively frequent, especially in infants, and may result from impairment of clearance mechanisms by the bacterium. Systemic manifestations of

disease may develop, associated with complications such as pulmonary atelectasis, bronchopneumonia, cyanotic paroxysms, and convulsions and, in rare instances, death (Halperin, 2004). Hypoxia from coughing may cause neurological complications, such as seizures and encephalopathy (a diffuse disorder of the brain), which are more common in infants.

A high percentage of all reported cases of pertussis require hospitalization, especially those in infants less than 6 months of age. A recent estimate of the case-fatality rate in the US was 0.2 % and the majority of these deaths (84%) were in infants < 6 months (Centers for Disease Control and Prevention: <http://www.cdc.gov/health/diseases.htm>).

1.3.3 Pathogenesis

It is believed that *B. pertussis* enters the respiratory tract in aerosol droplets produced by the coughing of an infected person. The organism is able to change from virulent to avirulent states by switching on and off the expression of groups of genes in response to specific environmental stimuli (Roberts and Parton, 2001). Pertussis is thought to be primarily a toxin-mediated disease. The bacteria attach to the respiratory cilia; using various factors such as FHA (Filamentous haemagglutinin), FIM (Fimbriae), PT (Pertussis toxin), TcfA (Tracheal colonization factor), BrkA (Bordetella resistance to killing), Vag8 (Virulence-activated gene-8 protein) and pertactin. They then produce toxins such as TCT (Tracheal cytotoxin), DNT (Dermonecrotic toxin) and CyaA (Adenylate cyclase) that paralyse the cilia, and cause inflammation of the respiratory tract, thus interfering with the clearing of pulmonary secretions and potentially causing pneumonia. Pertactin may act as an important adhesin but, in its absence, other proteins can perform this function (Mattoo and Cherry, 2005).

B. pertussis can evade host defences using its various virulence factors. With increased numbers of bacteria, local damage employing TCT, DNT, and perhaps CyaA develops consisting of ciliostasis, extrusion of dead ciliated cells, and infiltration of inflammatory cells. It is suggested that *B. pertussis* has the ability not only to protect itself, but also to inhibit the normal host reaction to other potential pathogens. Hewlett *et al.* (1985) demonstrated that children with culture-proven pertussis have a reversible impairment in delayed hypersensitivity responses to a battery of antigens, which is not seen in children with other bacterial infections.

1.3.4 Diagnosis

Early diagnosis is important for effective therapy and prevention of transmission. Although diagnosis of pertussis on clinical grounds is easily accomplished when a patient presents with a whoop and other characteristic features, diagnosis without classical signs and symptoms is problematic (Halperin, 2004).

In the past, laboratory diagnosis of these cases relied on conventional methods, including isolation in culture, direct detection of *B. pertussis* and/or *B. parapertussis* by direct fluorescent antibody (DFA) staining or by serological testing of paired acute and convalescent sera specimens. Isolation from respiratory specimens has a sensitivity and specificity of 73% and 85%-100% respectively, and often requires three to seven days for identification (Muller *et al.*, 1997). Gram staining, urease, oxidase, and nitrate reduction reactions usually can confirm identity. The direct fluorescent antibody test uses fluorescein-labelled antibodies to *B. pertussis* and *B. parapertussis* to detect the bacteria. The test is technically demanding, of low sensitivity and may give false negative results if the number of organisms in the specimen is low, or false positive results, due to lack of technical expertise, poor quality slides or antigenic cross-reactions with other organisms. Sensitivity and specificity of DFA are quite low (33% and 66%, respectively) with broad cross reactivity with normal microbial flora of the oropharynx and nasopharynx (Halperin *et al.*, 1989). Various serological tests such as agglutination, complement fixation and ELISA have been used to confirm the diagnosis of pertussis and parapertussis and are valuable in epidemiological and vaccine efficacy studies (Parton, 2003). Serological testing of sera of acute and convalescent specimens is a sensitive and specific epidemiological tool, but only yields retrospective information on infection.

Polymerase chain reaction (PCR) is the most rapid and sensitive detection method for *B. pertussis* and other fastidious, slow-growing pathogens. Several polymerase chain reaction (PCR) assays for detection of *Bordetella* DNA have been developed that demonstrate significantly greater sensitivity and specificity than either isolation or DFA testing provides. Primers have been designed to target four regions in the chromosome: (i) the promoter region of the genes encoding PT, (ii) a DNA segment upstream of the porin gene, (iii) repeated insertion sequences, and (iv) the adenylate cyclase toxin gene, *cyaA*. All except *cyaA* primers are specific for *B. pertussis* (Dragsted *et al.*, 2004). PCR for *Bordetella pertussis* has a sensitivity of 95.8%-98.9% and a specificity of 99.0%, which is dependent on the primers used and the gene targeted for amplification (Muller *et al.*, 1997). With the

advent of real-time PCR technology, identification can be completed within 1h after extraction of the DNA from a swab sample (Sloan *et al.* 2002). PCR methods provide the clinician with more timely and accurate information than that provided by isolation, DFA, or serological testing methods. The potential problem associated with PCR in the diagnosis of pertussis and other respiratory illnesses is false-positive results (Meade and Bollen, 1994). While the presence of *B. pertussis* and/or *B. parapertussis* DNA in a nasopharyngeal specimen strongly suggests active infection, the results should be used in conjunction with clinical presentation, patient history, and other diagnostic tests. When culture is associated with PCR and serologic testing, the greatest sensitivity occurs (Mattoo and Cherry, 2005).

1.3.5 Antimicrobial therapy

Decreasing the infectivity of the patient by protecting close contacts of that patient can limit spread of pertussis. Antibiotics given in the catarrhal stage may ameliorate the disease. However after the onset of paroxysms, antimicrobials have no apparent effect on the course of illness and are given mainly to limit the spread of the organisms to others. The drug of choice is erythromycin for 14 days, during the catarrhal stage of illness, which shortens the duration of symptoms and eliminates the organism from the upper respiratory tract within 5 days of initiation of therapy in most cases. Trimethoprim-sulfamethoxazole is a possible alternative for patients or contacts that do not tolerate erythromycin, and is effective in eradicating *B. pertussis* from the nasopharynx (CDC guidelines, 2005).

1.3.6 Vaccination and prevention (whole-cell and acellular pertussis vaccines)

Pertussis vaccination is considered to be more effective at preventing disease rather than infection due to the fact that the interepidemic period has not increased since the introduction of vaccination (Crowcroft and Britto, 2002). Pertussis vaccines used worldwide are generally whole-cell vaccines (WCVs). Vaccine potency, reactogenicity and the immune response that they produce, as well as the vaccination schedules, vary from country to country. Currently, worldwide pertussis vaccination coverage of children is about 80% (<http://www.who.int/vaccines/en/pertussis.shtml>).

Whole-cell vaccines (WCVs) and the more recently developed acellular vaccines (ACVs) are the two types of vaccines used. Whole-cell pertussis vaccines composed of killed whole *B. pertussis* organisms were developed in the 1940s, when the role of *B. pertussis* antigens

in immunity was still unclear, and have been used worldwide for many years. It was recognized early on that the production of serum antibodies, clinical protection and clinical toxicity were directly correlated with the number of organisms in the vaccine. In the mid-1940s, routine immunization of children with pertussis vaccines was started in the United States' and, during the 1950s, other countries also started performing routine pertussis immunization (Cherry, 1984). These proved to be highly efficacious but concerns about safety and reactogenicity led to the development of acellular pertussis vaccines in the 1970s (Mooi *et al.*, 2001).

Sato *et al.* (1984) in Japan first described the production of acellular pertussis component vaccines, which contained known antigenic components in measurable quantities. The first DTaP vaccines were developed at the Japanese National Institutes of Health in the late 1970s, where such vaccines were licensed for immunization of children aged two years or more in 1981, and for infants from the age of three months in 1989. All current acellular pertussis (aP) vaccines contain inactivated pertussis toxin which, in most cases, is combined with filamentous haemagglutinin and sometimes additional *B. pertussis* components such as fimbrial antigens and pertactin. The Ap vaccines are now available, either as individual vaccines or DTaP combinations, with or without the addition of hepatitis B, Hib, or poliovirus vaccine (IPV).

One monocomponent aP vaccine consists of PT alone, which has been toxoided by hydrogen peroxide treatment. PT and FHA are used for the two component aP and the addition of Prn is used for the three component aP. The five component vaccines contain the same antigens that are used for the three component aP with the addition of fimbriae 2 and 3. Most vaccines contain chemically toxoided PT (Hewlett, 1997). However, a third generation aP containing recombinant pertussis toxoid has been developed. This was considered to be an improvement over the chemically-toxoided PT as this treatment can change the structure and epitopes of molecules and reduce immunogenicity, thus requiring larger amounts of the antigen (Roberts and Parton, 2001). Field trials have shown the lower reactogenicity of ACVs (Jefferson *et al.*, 2003), but only one of them, containing PT, pertactin, Fim and FHA has revealed an efficacy level as good as WCV (Miller, 1999). The WCVs have therefore been replaced in some countries by ACVs because of their lower reactogenicity. ACVs provide some level of protection against pertussis and it seems that the more components an ACV contains, the greater the protection it provides (Hewlett, 1997).

DTaP causes moderate complications in less than 1% of injections, which include high fever greater than 105 degrees F (1 in 16,000 children), non-stop crying for more than 3 hours (1 in 1,000 children) and seizure (1 in 14,000 children) (WHO, 2003). Side effects and the probability of reactions seem to be highest in recipients of boosters in whom acellular vaccines were used for priming (Miller *et al.*, 2001). However, the aP vaccines are associated with immunological interference with other vaccines simultaneously administered at different sites. For example, the reduced immunogenicity of Hib vaccine when combined with some aP vaccines is of concern and needs further investigation (Mawas *et al.*, 2005; Mawas *et al.*, 2006).

The complete genome sequence data is now available for *B. pertussis* (Parkhil *et al.*, 2003) and it will give information on all the surface exposed and secreted proteins of an organism. This can lead to the identification of potential novel antigens from the genome sequence for possible addition to the next generation of acellular vaccines, a strategy known as reverse vaccinology (Preston and Maskell, 2002).

1.3.7 Re-emergence of pertussis

Pertussis is an endemic disease even with the widespread use of vaccination, with frequent epidemic outbreaks. Mass vaccination has greatly reduced the incidence of pertussis; however, the disease is re-emerging even in some countries with high vaccination coverage. The resurgence of pertussis in countries such as the Netherlands, the United States, Canada and Australia has been studied to find an explanation for this re-emergence.

In spite of mass vaccination against *B. pertussis* in the Netherlands since 1953, outbreaks of pertussis occurred in 1996, 1999 and 2001, when the vaccine coverage rate was 96% for at least three vaccinations in the first year of life. During the inter-epidemic years, the incidence of the disease did not return to levels observed before 1996. It has been suggested that *B. pertussis* variants in genes such as PT or pertactin may be responsible for a reduced efficacy of currently-used vaccines and an associated re-emergence of pertussis among vaccinated populations. Another postulate for re-emergence is that the role of herd immunity due to high levels of pertussis vaccination may influence endemic *B. pertussis* populations via selective pressure for strains which contain variants of genes (Byrne and Slack, 2006). The antigenic divergence between vaccine strains and clinical isolates has also been detected in other countries such as the US and Finland with high vaccine coverage (Mooi *et al.*, 1999; Cassiday *et al.*, 2000). Other possibilities for re-emergence might be

changes in surveillance (accuracy of notifications) and diagnostic criteria, changes in vaccine quality, decrease in vaccine coverage or waning vaccine-induced immunity.

1.4 *Bordetella pertussis*

1.4.1 Virulence factors

A pathogen is able to enter the host and interacts with a specific target tissue, evades host defences to survive, develops localized damage at the site of infection and may disseminate either itself or its products to cause systemic disease by its virulence factors. Many of the virulence factors characterized in the bordetellae are common to *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*. These consist of adhesins, such as filamentous hemagglutinin (FHA), pertactin, tracheal colonization factor and fimbriae, and toxins, such as adenylate cyclase haemolysin, dermonecrotic toxin and tracheal cytotoxin (Fig. 1.2). However, there are other virulence factors, which are expressed by just one of the species; such as pertussis toxin and serum resistance to killing protein (BrkA) expressed by *B. pertussis* or a type III secretion system expressed by *B. bronchiseptica* (Parkhill *et al*, 2003).

One of the strategies used in the recognition of virulence factors of pertussis include application of a modified form of Koch's postulates (for example, the factor is produced only by virulent organisms and the purified factor produces effects analogous to the disease), by the characterization of mutant strains developed using transposon-mediated mutagenesis, and the use of bacterial challenge in immunized animals. However, this last approach is restricted, since a protective antigen is not necessarily a virulence factor. There are various possible virulence factors of *B. pertussis* (Kerr and Matthews, 2000).

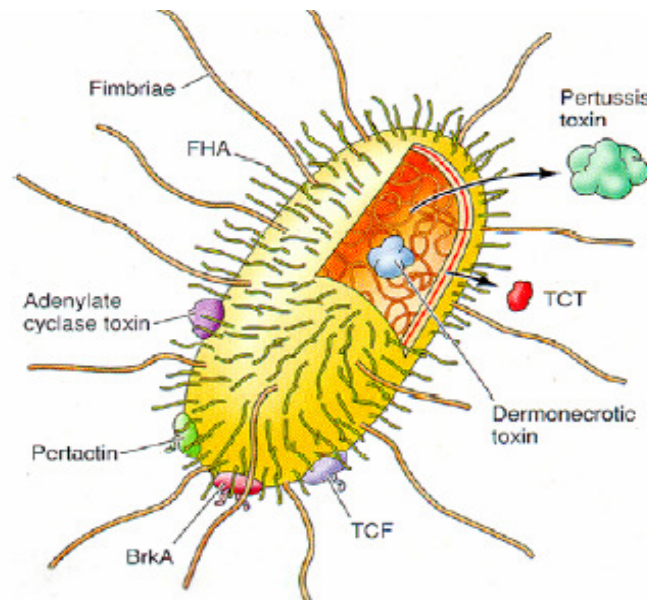


Figure 1.2 Virulence factors of *Bordetella pertussis* (adapted from www.molgen.uc.edu/_mediaPDF/faculty/alison.weiss.pdf.)

1.4.1.1 Adhesins

Adhesion to host cells is critical to the initiation of infection by most bacterial pathogens. *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* exhibit tropism for ciliated epithelia in the respiratory tract (Weiss and Hewlett, 1986; Soane *et al.*, 2000). Bacterial replication leads to a great increase in the number of *B. pertussis* attached to the cilia and is coupled with the loss of ciliated and non-ciliated cells. *B. pertussis* produces, under the control of the BvgA/S system (see section 1.4.3), a number of surface adhesin molecules including fimbriae, also called agglutinogens, filamentous haemagglutinin, pertactin, the serum-resistance protein BrkA and the attachment factor Tcf.

1.4.1.1.1 Fimbriae

B. pertussis has two related but genetically-distinct serotypes, serotype 2 and serotype 3, due to expression of the major fimbrial subunits Fim2 (22.5 kDa) and Fim3 (22kDa) respectively. The *bvg* locus positively regulates their expression (Zhang *et al.*, 1985; Mooi, 1994). In addition to the major subunits, the fimbriae also have the minor 40-kDa subunit FimD, at the tip of both types of fimbriae that acts as the adhesin (Willems *et al.*, 1993; Geuijen *et al.*, 1997). A periplasmic chaperone, FimB and an outer-membrane usher protein, FimC are required for the assembly of *B. pertussis* fimbriae.

Introduction

In addition to *bvg* regulation, expression of the *fim2* and *fim3* genes is also regulated by mutations in their promoter regions, which leads to serotype switching (Willems *et al.*, 1990). In particular, a C-rich region may experience relatively frequent deletions or insertions, which strongly alters the transcription of the fimbrial genes. Therefore, some strains produce both types of fimbriae (serotype 2, 3), some only one (either serotype 2 or 3), or some none at all (Locht, 1999). The genes *fim2*, *fim3*, *fimX* (a homologous gene, whose product has not been identified); and a recently identified fourth fimbrial locus, *fimN* (in *B. bronchiseptica*) (Kania *et al.*, 2000) are single genes on the chromosome (Stibitz and Garletts, 1992), but the *fimBCD* genes are clustered together with the *fha* and *bvg* genes and compose a polycistronic operon with *fhaC* (Willems *et al.*, 1994). It has been proposed that FimB and FimC function as a chaperone and usher, respectively, similar to the *E. coli* PapD and PapC (Willems *et al.*, 1992). The arrangement of those genes, which are necessary for the production of Fim2 and Fim3 and the structural gene for the adhesive tip protein (FimD), is *fimABCD*. The first gene, *fimA*, is not expressed in *B. pertussis* because of a deletion at its 5' end. In contrast, intact *fimA* genes are present in *B. bronchiseptica* and *B. paraptussis*, and *fimA* is expressed in *B. bronchiseptica*, although its function has not been discerned (Locht *et al.*, 1992; Willems *et al.*, 1992; Boschwitz *et al.*, 1997).

The major fimbrial subunit, like FHA (see below), can bind sulphated glycoconjugates similar to heparan sulphate on the surface of epithelial cells (Geuijen *et al.*, 1998). The minor subunit FimD attaches to the VLA-5 integrin of monocytes and macrophages. The binding of *B. pertussis* to VLA-5 upregulates CR3 (Complement Receptor type III) (Menozzi *et al.*, 1991) integrin expression, and consequently increases the attachment of the organism via FHA to macrophages (Hazenbos *et al.*, 1995) (section 1.4.1.1.2). Thus, the fimbriae and FHA may both assist in *B. pertussis* adhesion to, and perhaps invasion of, alveolar macrophages. Some studies in animal models using *B. pertussis* mutants have also suggested that FHA and fimbriae collaborate in the colonization of the trachea, which offers some biological insight to the tightly coordinated expression of these two adhesins. The binding affinity of fimbriae is determined by both the degree and arrangement of sulphate groups on the cell surface receptors (Yanagishita *et al.*, 1992). It has also been suggested that fimbriae play a minor role in biofilm formation (Irie *et al.*, 2004).

Fimbriae are highly immunogenic and stimulate the production of agglutinating antibodies (Robinson *et al.*, 1986). The fimbriae were initially termed agglutinogens in view of the fact that anti-fimbrial antibodies in the sera of infected or vaccinated children agglutinated the bacteria. Furthermore, a correlation between the presence of agglutinating antibodies and

protection (Preston, 1976), supported the inclusion of Fim2 and Fim3 as antigens in some of the new acellular pertussis vaccines. The interactions of FIM with epithelial cells and monocytes/macrophages play an important role not only in adherence but also in the degree and nature of the immune response to pertussis (Mattoo and Cherry, 2005).

1.4.1.1.2 Filamentous haemagglutinin

The best-studied *B. pertussis* adhesin is filamentous haemagglutinin (FHA). It appears to be the major adhesin of *B. pertussis* and one of the two haemagglutinins (the other being PT) and its primary role appears to be in colonization of the ciliated epithelium (Parton, 2003). It is produced as a 367-kDa precursor encoded by the *fhaB* gene, which is positioned directly upstream of the *bvg* genes (Locht *et al.*, 1993). The N-terminal and C-terminal proteolytic processing of the FhaB precursor leads to the 220-kDa mature FHA (Renauld-Mongénie *et al.*, 1996). Transport across the inner membrane is directed by a 71 amino acid signal sequence, which is then removed. Transport across the outer membrane requires an accessory outer membrane protein FhaC (Jacob-Dubuisson *et al.*, 2000), whose gene is placed directly downstream of the structural *fhaB* gene. Eventually, the C-terminal portion is cleaved by the surface bound serine protease SphB1 (Coutte *et al.*, 2001) (see below) and the N-terminal 220-kDa mature FHA is released.

FHA is a filamentous structure about 2 nm wide and 50 nm long, folded into a monomeric inflexible rod based on a 19-residue repeat motif, which is rich in beta strands and turns (Makhov *et al.*, 1994). The critical step in colonization and infection of the upper respiratory tract in pertussis is the adherence of *B. pertussis* to ciliated cells and macrophages, which requires the recognition of eukaryotic carbohydrates or integrins by FHA. Hence, three different domains of the mature FHA are involved in the differential recognition of receptors on host cells: (1) the first part, consisting of residues from 1 to 400 are responsible for binding to sulphated sugars on mucus-secreting epithelial cells (Hannah *et al.*, 1994), (2) a fragment including residues 1097 to 1099, the RGD sequence, binds to CR3 integrins on macrophages and ciliated cells (Locht *et al.*, 1993), (3) A region from 1141 to 1279, the carbohydrate recognition domain (CRD), is responsible for binding to lactosylceramides on macrophages and ciliated cells (Prasad *et al.*, 1993), although it is not important for uptake of *B. pertussis* into macrophages. It has also been reported that the RGD motif of FHA binds to very late antigen 5 (VLA-5; an $\alpha_5\beta_1$ -integrin) of bronchial epithelial cells (Ishibashi *et al.*, 2001), which consequently induces activation of NF- κ B, followed by an up-regulation of the epithelial intercellular adhesion molecule 1 (ICAM-1)

(Ishibashi and Nishikawa, 2002). This up-regulation leads to the accumulation and activation of leukocytes at the site of bacterial infection.

In the absence of opsonizing antibodies, FHA-mediated binding of *B. pertussis* to macrophages via CR3 leads to phagocytosis that does not result in subsequent killing and may be critical in the intracellular survival of this organism by enabling the bacteria to avoid triggering an oxidative burst and to survive and multiply intracellularly (Relman *et al.*, 1990). FHA also binds the integrin sites that are important for recognition of C3bi, coagulation factor X, and endothelial cells. It can also inhibit the production of IL-12 by macrophages in response to LPS and γ -IFN, by stimulating the production of IL-10 (McGuirk and Mills, 2000). Interaction of FHA with monocytes/macrophages has also been shown to induce apoptosis and to inhibit antigen-dependent CD4⁺ T cell proliferation (Abramson *et al.*, 2001). FHA in *B. bronchiseptica* has been shown to play a critical role in immunomodulation and a role in host specificity (Inatsuka *et al.*, 2005).

FHA provokes a strong antibody response, in both humans and animal models, both systemically and mucosally (Thomas *et al.*, 1989), and vaccination with purified FHA protects against respiratory challenge in mice. Therefore, FHA was incorporated in second-generation acellular vaccines against whooping cough (Sato *et al.*, 1984). It has been reported that acellular vaccines that contain FHA as well as PT toxoid have slightly greater efficacy than monocomponent PT toxoids (Cherry and Heininger, 2004). FHA also has adjuvant activity for antigens delivered with it by the nasal route (Poulain-Godefroy *et al.*, 1998). These properties have been considered for developing systems for presenting heterologous antigens to the epithelium of the respiratory tract (Renauld-Mongénie *et al.*, 1996).

1.4.1.1.3 Pertactin

The autotransporter pertactin is a Bvg-regulated surface-located and secreted protein. This was initially referred to as P69 or 69-kDa protein, which reflected its apparent molecular weight and processed from a 93-kDa large precursor (Charles *et al.*, 1994), as determined by SDS-PAGE. The actual size of the processed form is estimated to be 61 kDa, and the β -domain is 32 kDa. The protein is a virulence factor, and a member of the pertussis autotransporter proteins family (see section 1.5), and mediates adhesion to mammalian cells, a reaction mediated partly by an RGD sequence.

The X-ray crystal structure of P.69 pertactin has been solved to 2.5Å (the only autotransporter protein for which the crystal structure has been determined). The protein structure demonstrates a 16-strand parallel β -helix (the largest β -helix known to date) with a V-shaped cross-section. A number of weakly conserved amino acid repeats form internal and external ladders. Several loops project from the helix and contain sequence motifs that are responsible for the biological activity of the protein. A (GGXXP)₅ sequence motif is positioned directly down from the RGD motif and may mediate interaction with epithelial cells, while the C-terminus region of P.69 pertactin contains a (PQP)₅ motif loop with a major immunoprotective epitope (Emsley *et al.*, 1996).

It has been noted that pertactin is polymorphic between the various *Bordetella* species, and even between different strains within the same species (Mooi *et al.*, 1998). Pertactin in *B. pertussis* (Charles *et al.*, 1989), *B. parapertussis* (Li *et al.*, 1991), and *B. bronchiseptica* (Li *et al.*, 1992) corresponds to proteins of 69 kDa (P.69), 70 kDa (P.70), and 68-kDa (P.68), respectively. The different number of internal proline-rich tracts and leucine-rich repeats within the proteins explain the difference in size between mature P.68, P.69, and P.70, where the P.68 has the fewest and P.70 the most (Henderson and Nataro, 2001). These motifs are frequently present in molecules that are responsible for protein-protein interactions in eukaryotic cells (Emsley *et al.*, 1994).

Pertactin is produced at an intermediate phase, *in vitro*, during *B. pertussis* growth, after FHA and before pertussis toxin production (Kinnear *et al.*, 1999). It supports the idea that, *in vivo*, FHA performs as an early adhesin molecule and pertactin encourages more intimate adhesion to mammalian cells at a following step and earlier to toxin release.

During the development of a veterinary vaccine against *B. bronchiseptica*, pertactin was discovered (Novotny *et al.*, 1985) and it was found that it provided protection in animal models. However, mutant strains lacking the pertactin protein appeared to colonize the mouse respiratory tract as efficiently as the wild type parent strains, although mutant strains deficient in both pertactin and FHA were shown to be cleared much more quickly than the wild-type parent strains (Locht, 1999). This supports the idea that pertactin is involved in bacterial adhesion. The protective effect of pertactin in animal models and its involvement in adhesion has targeted it as one of the antigens included in some of the new acellular pertussis vaccines (Gustafsson *et al.*, 1996).

1.4.1.1.4 *Bordetella* resistance to killing protein (BrkA)

The autotransporter BrkA protein is a *B. pertussis* virulence factor that confers serum resistance and also operates as an adhesin (Fernandez and Weiss, 1994). Fernandez and Weiss identified an insertional mutant of *B. pertussis*, from a panel of strains with mutations in *bvg*-regulated genes caused by insertion of the transposon Tn5 *lac* (a transposon which possesses a promoterless β -galactosidase (β -Gal) gene, which is expressed only if the transposon inserts downstream of a functional promoter) which was at least 10-fold more susceptible to serum killing than the wild type (Fernandez and Weiss, 1994) and was less virulent in an infant mouse model where approximately 10^7 bacteria (CFU) were administered intranasally (Weiss and Goodwin, 1987). Serum sensitivity was assessed *in vitro* by exposing strains to 20% normal human serum for 60 min at 37°C, using 10^7 organisms, followed by diluting the organisms 10-fold in phosphate-buffered saline (PBS) containing 10 mM EDTA to halt the complement activity (Fernandez and Weiss, 1994).

The locus encoding this serum resistance function encodes two divergently-transcribed open reading frames, named BrkA and BrkB. Although both ORFs were originally thought to be essential for serum resistance (Fernandez and Weiss, 1994), the results observed for a BrkB mutant in its serum resistance were not statistically significant (Fernandez and Weiss, 1998). Putative sites for BvgA binding are located within the 300 bases that separate the two ORFs. A third ORF is about 100 bases downstream of *brkA*, encoded on the complementary strand, is not necessary for serum resistance, and is homologous at the amino acid level to the heat shock protein-chaperonin Cpn60. BrkA shows an overall 29% sequence identity to pertactin and has two RGD motifs, a conserved proteolytic processing site and an outer membrane-targeting signal (Fig. 1.3). Like pertactin, BrkA has been shown to be involved in adherence to mammalian cells (see below). Despite the similarities, a pertactin mutant was not as sensitive to serum killing as the BrkA mutants. BrkB is comparable to ORFs of unknown function in *E. coli* and *Mycobacterium leprae* and is considered to be a cytoplasmic membrane protein (Fernandez and Weiss, 1994).

BrkA is expressed as a 103-kDa precursor and like pertactin, contains outer-membrane localization signals and an approximately 300-residue C-terminal autotranslocator domain, which has 54.5% identity to that of pertactin (Fig. 1.3), suggesting that BrkA, like pertactin, may use a similar secretion mechanism (Locht, 1999). Recent analysis of BrkA with SignalP V2.0 (Nielsen *et al.*, 1998) has predicted a 42-amino-acid signal peptide. The 103-kDa precursor is processed during secretion to yield a 73-kDa N-terminal (passenger) α -

Introduction

domain and a 30-kDa C-terminal β -domain (Shannon and Fernandez, 1999). The cleaved 73-kDa BrkA passenger remains firmly associated with the bacterial surface (Oliver and Fernandez, 2001; Oliver *et al.*, 2003a) and cannot be detected in culture supernatants of *B. pertussis* (Oliver and Fernandez, 2001). The BrkA C-terminal domain is able to form a pore that supports the proposed model of autotransporter export (Shannon and Fernandez, 1999). The processing site, using the outer membrane fractions isolated from *B. pertussis*, lies between residues Asn₇₃₁ and Ala₇₃₂ (Passerini *et al.*, 1999).

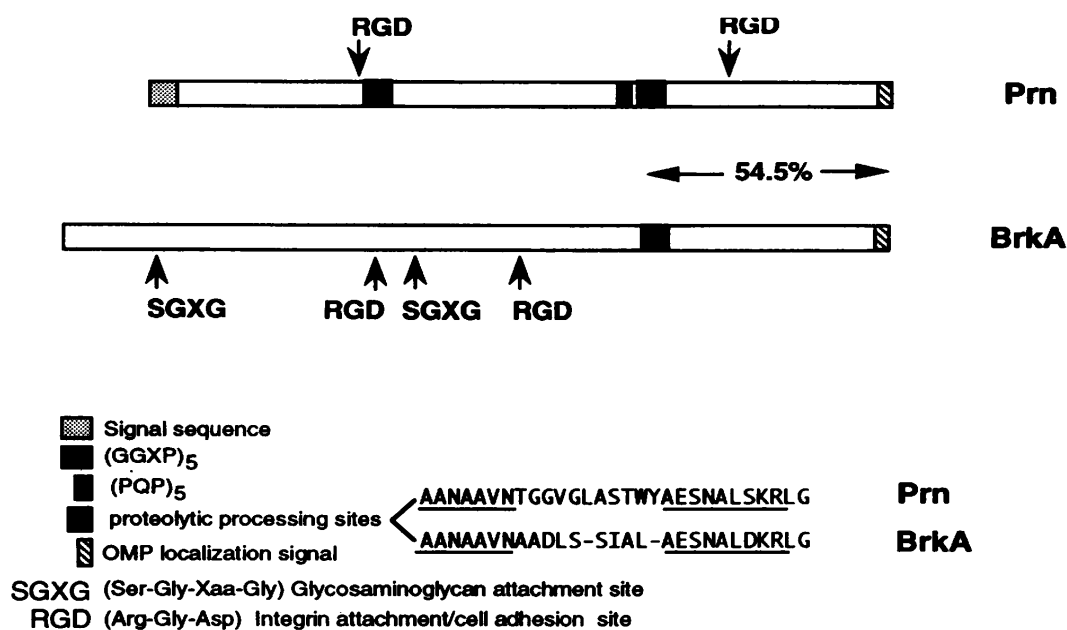


Figure 1.3 Structural comparison of BrkA and pertactin (Prn). The overall sequence identity of 29% rises to 54.5% at the C terminus. X and Xaa refer to any amino acid (Adapted from Fernandez and Weiss, 1994).

It was shown recently that a region in the C-terminus of the BrkA passenger domain is necessary for its folding simultaneously with or subsequent to the translocation via the β -domain channel (Oliver *et al.*, 2003b) (see TypeV secretion system, section 1.6.5). This 31- to 39-amino-acid region, termed the linker region, is located immediately upstream of the β -domain and, using deletion analysis, was found to be essential for surface expression of BrkA. This linker region, together with the β -domain, is characterized as the minimal BrkA translocation unit. Moreover, the linker region may also anchor the BrkA α -domain to the bacterial surface (Oliver *et al.*, 2003b). This conserved region can be observed in a large

group of autotransporters with various functions, which indicates that it provides an important function for secretion of autotransporters.

Several features in BrkA suggest that it is involved in adhesion to host cells. It contains two Arg-Gly-Asp motifs (RGD), which bind to integrin receptors on host cells, and two putative binding sites for sulphated glycoconjugates (SGXG), which could possibly imitate the FHA-heparin interaction (Fig. 1.3). It has been shown *in vitro* that the binding of a BrkA mutant strain to eukaryotic cells (human lung fibroblast cell line MRC-5) is about half of the level of the wild-type in a 3-h assay and, in mice challenged intranasally. The mutant is also less virulent than the wild-type parent strain in terms of lung colonization at 7 days (Fernandez and Weiss, 1994).

Possible mechanisms of serum resistance have been suggested on the basis of the predicted amino acid sequences of BrkA. The outer membrane localization signal (Struyvé *et al.*, 1991) on BrkA suggests that it is targeted to the surface of *B. pertussis*, and it may act to prevent or block the binding of lytic antibody to a bactericidal target, or it could draw blocking antibody, which would present a similar function. Alternatively, the putative glycosaminoglycan attachment site (SGXG) (Fig. 1.3) on BrkA may control serum resistance. A heparin- (glycosaminoglycan) binding site on the serum protein vitronectin acts to inhibit the polymerization of the complement component C9 (Milis *et al.*, 1993). It is supposed that BrkA could possibly utilize its putative heparin-binding site to prevent C9 polymerization. The Rck protein mediates serum resistance in *Salmonella typhimurium* by a mechanism that inhibits the polymerization of the C9 protein of complement (Heffernan *et al.*, 1992).

It has also been suggested that BrkA inhibits activation or promotes degradation of C4 after deposition (Barnes and Weiss, 2001). This could occur either by preventing complement function or by recruiting a complement-inhibitory protein. BrkA could prevent complement function by acting as a protease against C1r, C1s, or C4, which would inactivate the C1r or C1s and inhibit deposition of C4. On the other hand, proteolysis of activated C4 would also result in decreased C4 deposition (Barnes and Weiss, 2001). Another possibility is that BrkA may bind to and activate a complement inhibitor. There are lots of inhibitors of the complement cascade but only four operate at the C1/C4 step, which are soluble C4 binding protein, C1 inhibitor, membrane-bound CD46 (membrane cofactor protein) and CR1. The likeliest binding partners for BrkA are the soluble complement regulators (Barnes and Weiss, 2001). It has been shown that purified C4 binding protein binds to filamentous

haemagglutinin (FHA) (Berggard *et al.*, 1997). However, FHA is not an essential molecule for resistance to complement (Fernandez and Weiss, 1998), suggesting that C4 binding protein bound to FHA is not protective. It has been shown that deposition of C4 binding protein was greatly reduced on a FHA mutant, and less deposition was detected on a *bvg* mutant, which could not express all virulence factors, such as BrkA. Therefore, this additional decrease in binding may possibly indicate that BrkA can also bind C4 binding protein and this binding of C4 to BrkA may cause serum resistance (Barnes and Weiss, 2001).

Another possible mechanism of action could be the presence of two SGXG proteoglycan binding motifs on BrkA (Fernandez and Weiss, 1994), which permit efficient binding of the highly glycosylated C1 inhibitor (Bock *et al.*, 1986). Thus, the accumulation of C4 on the surface of the bacteria would be decreased via recruitment of either C4 binding protein or C1 inhibitor by BrkA.

BrkA can therefore protect the bacterium, by inhibiting the activation of the complement cascade and this would have obvious benefits in the human respiratory tract where complement can be found. Inhibition of immune responses has obvious benefits for the bacterium (Barnes and Weiss, 2001). However, it has been shown that antibody alone is usually effective at opsonizing bacteria and promoting phagocytosis; adenylate cyclase toxin of *B. pertussis* (section 1.4.1.2.2) interferes with the process of opsonization with antibodies, as it inhibits phagocytosis by human neutrophils (Towbin *et al.*, 1979).

Southern blot analysis showed the presence of *brkAB* sequences in *B. bronchiseptica* and *B. parapertussis* but not in *B. avium*. Clinical isolates of *B. bronchiseptica* and *B. parapertussis* were also shown to be serum resistant, and wild-type strains with extra copies of the *brk* locus were two- to fivefold more resistant to serum killing (Fernandez and Weiss, 1998). BrkA conferred resistance to killing by complement in a BrkA-deficient mutant strain of *B. pertussis*, but sensitivity to complement-mediated killing was not observed in a *B. bronchiseptica* BrkA mutant strain. This may possibly be due to the fact that *B. bronchiseptica* is naturally more resistant to complement than *B. pertussis* due to its LPS composition or because *B. pertussis* has an abbreviated O-antigen structure (Rambow *et al.*, 1998) (see section 1.4.1.2.5).

BrkA is not a current component of any acellular pertussis vaccines although its role as a possible adhesin molecule and in mediating protection from complement suggests that it

might be considered for inclusion in any future generations of acellular pertussis vaccines. It has been shown recently that both the purified 73 kDa N-terminal α -domain of BrkA combined with Cpn60 chaperonin from *B. pertussis* soluble fractions stimulated antibodies in mice and induced a 42% protection rate against intracerebral challenge with live *B. pertussis* strain 18323 (Cainelli Gebara *et al.*, 2007). Since acellular vaccines, unlike the generally more protective whole-cell vaccines, do not protect against intracerebral challenge in mice, these findings suggest that BrkA/Cpn60 might be useful additional components of acellular vaccines.

1.4.1.1.5 Tracheal colonization factor

Tracheal colonization factor A (TcfA) is produced by *B. pertussis* but not by *B. bronchiseptica* or *B. parapertussis* strains. The protein structure of TcfA is similar to pertactin, and seems to be particularly involved in the colonization of the trachea. When mice were challenged in a competition assay, with wild-type and a TcfA mutant strain of *B. pertussis*, the number of mutant bacteria isolated from the trachea was decreased 10-fold compared with the number of the wild-type strain (Finn and Stevens, 1995). The mechanism involved in the observed difference has yet to be clarified.

The predicted amino acid sequence of TcfA shows a 68-kDa RGD-containing, proline-rich protein (Finn and Stevens, 1995). The C-terminal end of unprocessed TcfA is an autotransporter domain with a high degree of sequence similarity to those of pertactin and BrkA, suggesting a similar secretion mechanism. The C-terminus has a 50% amino acid identity to the pertactin β -domain (Finn and Stevens, 1995). The 34-kDa N-terminal α -domain of TcfA contains an RGD motif presumed to be involved in integrin binding, like those of FHA, pertactin and BrkA. Immunoblots of *B. pertussis* whole-cell lysates with polyclonal antiserum raised against a part of the N-terminal portion of TcfA identified 90- and 60-kDa bands. Nevertheless, culture supernates of *B. pertussis* contained only the 60-kDa form, suggesting the presence of outer-membrane-associated and secreted forms of the α -domain (Henderson and Nataro, 2001).

1.4.1.2 Toxins

Apart from lipopolysaccharide, also called endotoxin, and the tracheal cytotoxin, which is a fragment of the *Bordetella* peptidoglycan, the other known *B. pertussis* toxins are

proteins. The three major protein toxins are pertussis toxin, adenylate cyclase toxin and dermonecrotic toxin (Locht, 1999).

1.4.1.2.1 Pertussis toxin (PT)

Pertussis toxin (PT) is an exotoxin and a main colonization factor. It is released into the extracellular milieu and also remains cell-bound. PT is a 105 kDa hexamer, consisting of an A subunit, the S1 polypeptide (MW 26,026) and a complex pentamer B subunit comprising S2 (MW 21,925), S3 (MW 21,873), S4 (MW 12,059) and S5 (MW 11,013) polypeptides in a molar ratio of 1: 1: 2: 1 (Tamura *et al.*, 1982; Saukkonen *et al.*, 1992). The S1 subunit of PT, also called the A protomer, is responsible for most of the biological activities of the toxin and has enzymatic activity. The S2 to S5 subunits are responsible for the interaction of the toxin with the receptors of target cells by forming the B moiety of the toxin. There is a significant structural homology between the A and B subunits of PT and other bacterial toxins like cholera toxin of *Vibrio cholerae*, shiga toxin of *Shigella dysenteriae* and the heat-labile toxin of *E. coli* (Madan Babu *et al.*, 2001).

PT is expressed by *B. pertussis* and is actively secreted. The full set of structural genes for the toxin subunits, *ptx*, and for toxin translocation, *ptl*, are also present in the *B. parapertussis* and *B. bronchiseptica* genomes (Marchitto *et al.*, 1987). However, it has been shown to be transcriptionally silent due to the existence of defective promoters or activator (BvgA) binding sites (Arico and Rappuoli, 1987). All the toxin subunits are individually translocated through the inner membrane, via classical signal peptides. The subunits assemble into the holotoxin structure inside the periplasm, after the removal of signal peptides, and then the assembled toxin is secreted into the extracellular milieu of *B. pertussis*. Nine accessory proteins encoded by the *ptl* genes, which are positioned immediately downstream from the five structural *ptx* genes, are required for this last step in toxin secretion (Weiss *et al.*, 1993). They are cotranscribed with the *ptx* genes, in a single polycistronic operon. As with many other virulence genes of *B. pertussis*, the activation of the *ptx* operon is Bvg-dependent (Boucher *et al.*, 1997).

The molecular action of the toxin can be separated into three main steps, (i) the toxin becomes attached to target cell receptors by the B oligomer, (ii) the S1 subunit is translocated into the cytosol, and (iii) the enzyme activity of the translocated S1 subunit is activated (Locht and Antoine, 1995). ADP-ribosyltransferase activity in the S1 subunit employs NAD for signal transduction, and G proteins in target cells as ADP-ribosyl

acceptors. The toxin has a diversity of pathophysiological effects, depending on the affected cell type, such as histamine sensitization, activation of insulin secretion, or lymphocytosis, hallmarks of the whooping cough syndrome (Munoz, 1985). However, it has been suggested that this toxin is probably not directly responsible for the typical paroxysmal cough and vomiting which are characteristic of pertussis (Locht, 1999). Recently, in the rat, it was shown that the PT caused relaxation of small arteries without causing vascular lesions or inducing morphological or numerical changes in cell organelles such as contractile elements of vascular smooth muscle cells (van Meijeren *et al.*, 2004).

After human infection with *B. pertussis* or vaccination with classical whole-cell vaccine, high levels of antibody to PT can be detected (Thomas *et al.*, 1989). Moreover, the toxin alone, in toxoid form, provides protection against challenge in mouse models (Locht, 1999). Today, inactivated PT is therefore the major protective antigen in all the available acellular vaccines. More recently, plasmid DNA expressing the S1 subunit of PT was evaluated for immunogenicity and for the ability to induce protection against PT challenge or *B. pertussis* infection in mice. This DNA vaccination induced anti-PT IgG antibody production, inhibited leukocytosis-promoting activity and induced protection against intracerebral challenge with *B. pertussis* (Kamachi *et al.*, 2003).

1.4.1.2.2 Adenylate cyclase toxin (CyaA)

Adenylate cyclase toxin (CyaA), an important invasive toxin secreted by *B. pertussis*, is a bifunctional calmodulin-sensitive adenylate cyclase/haemolysin. The *cya* operon of *B. pertussis* encodes the adenylate cyclase toxin and contains five genes: *cyaA*, *B*, *C*, *D* and *E* (Figure 1.4). The *cya* operon is under the regulation of the *bvg* locus and expressed maximally in the Bvg⁺ phase. Adenylate cyclase is secreted without proteolytic processing which is different from other *Bordetella* virulence factors. Three accessory proteins, CyaB, CyaD and CyaE, the genes for which are positioned directly downstream of the adenylate cyclase structural gene (*cyaA*), are responsible for toxin secretion (Glaser *et al.*, 1988). A single promoter controls the expression of *cyaA*, which encodes the toxin. This region binds BvgA phosphate to a widespread sequence covering positions from -137 to -51 on the promoter upstream to the transcriptional start site. This part includes several heptameric variants of the BvgA-binding consensus 5'-TTTCCTA-3'. A second promoter, located between *cyaA* and *cyaB* genes, controls the expression of *cyaB*, *D* and *E*. The gene *cyaC* is transcribed in the opposite direction to the other genes in the *cya* operon and the *cyaC* product is essential for the palmitoylation of CyaA at Lys 983 in terms of converting it into

the active form (Glaser *et al.*, 1988). Secretion of a functional CyaA requires the products of all these genes (Glaser *et al.*, 1988).

CyaA has two activities, adenylate cyclase enzymatic activity and pore-forming/haemolytic activity, which are on different domains of the protein (Fig. 1.5). The pore forming/haemolytic domain enables the enzymatic domain to enter into the target cell. The entry of the CyaA toxin into eukaryotic cells is performed in the following order: (i) the toxin binds to the target cell surface by interacting with integrin receptor CD11b/CD18 (Guermónprez *et al.*, 2001) but in the absence of this receptor can also penetrate lipid bilayers (Martin *et al.*, 2004). Binding is dependent on the palmitoylation of Lys 983. (ii) The haemolytic domain of the toxin makes a pore or channel (Iwaki *et al.*, 2000). Subsequent to its entry into the cell, the toxin demonstrates adenylate cyclase activity which is activated in eukaryotic cells by calmodulin (Ladant, 1988). It generates almost a thousand-fold increase in intracellular cAMP levels in phagocytic cells which are thought to be the primary target, and inhibits their various chemotactic, phagocytic and bactericidal activities (Mooi, 1988).

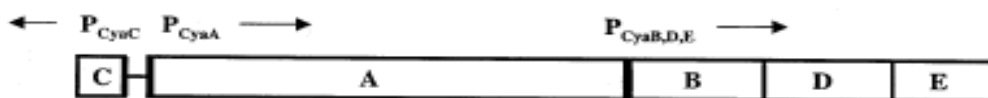


Figure 1.4 The *cya* operon. The *cyaC* gene is controlled by promoter P_{CyaC} and transcribed in the opposite direction to that of the other genes in the *cya* operon (Adopted from Madan Babu *et al.*, 2001).

The N-terminal portion of the toxin contains the catalytic domain. Multiple GGXGXDXLX repeats (Fig. 1.5) in the C-terminal portion makes adenylate cyclase toxin a member of the RTX (repeats-in-toxin) family, a group of calcium-dependent, pore-forming cytotoxins. Calcium ion may bind to these repeats as an essential ion for toxin activity (Glaser *et al.*, 1988).

It has been shown that adenylate cyclase mutant strains of *B. pertussis* colonized the respiratory tract of infected mice much less than the wild-type strains (Weiss *et al.*, 1984). Moreover, CyaA-deficient mutants were not able to produce lethal infection in infant mice, unlike wild-type strains (Weiss and Goodwin, 1989). Also, the adenylate cyclase toxin is

Introduction

responsible for cell death, via apoptosis, of alveolar macrophages that come into contact with *B. pertussis*. This phenomenon may possibly be a main way for the organism to escape the innate immune system of the infected host, and enable it to efficiently colonize the host. For the initiation of apoptosis, both enzymic and pore-forming activities are required (Khelef *et al.*, 1992). It has been suggested therefore that CyaA acts primarily as an antiphagocytic and anti-inflammatory factor during infection. It has also been shown that the *B. bronchiseptica* adenylate cyclase toxin (ACT) and a type III secretion system (T3SS) modulate multiple signal transduction pathways resulting in bacterial persistence (Skinner *et al.*, 2005). ACT and T3SS disrupt cell-signalling pathways resulting in production of immunosuppressive cytokines and the systemic suppression of IFN- γ production (Reissinger *et al.*, 2005).

It has been shown that adenylate cyclase toxin from *B. pertussis* protected infant mice against lethal challenge and also shortened the duration of lung colonisation challenged 14 days after vaccination (Guiso *et al.*, 1989; Guiso *et al.*, 1991). The purified active CyaA, either the native form from *B. pertussis* or the recombinant form, also showed protective activity in mice against intranasal challenge with *B. pertussis* (Hormozi *et al.*, 1999).

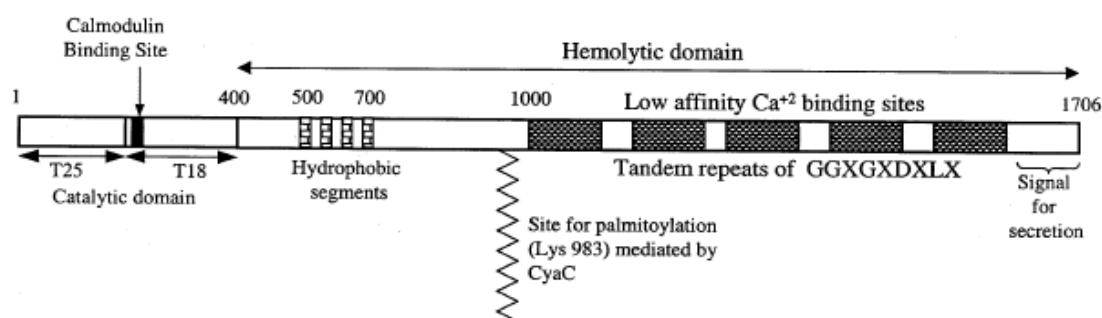


Figure 1.5 Structural organization of the CyaA toxin. The catalytic domain extends from 1 to 400 amino acid residues, and contains the T25 and T18 subdomains. The T18 domain includes the calmodulin-binding site. The pore-forming haemolytic domain extends from amino acids 401 to 1706 and contains Lys 983 (required for activation by palmitoylation mediated by CyaC) and calcium binding sites with tandem repeats of the sequence GGXGXDXLX (Adapted from Ladant and Ullmann, 1999).

More recently, it has been shown that the adenylate cyclase of *B. pertussis* can act as an adjuvant for other antigens, particularly a form of CyaA lacking enzymatic activity (CyaA*). The CyaA* form enhanced protection when co-administered intraperitoneally with an acellular pertussis vaccine and this enhancement was associated with an augmentation of both Th1 and Th2 immune responses to *B. pertussis* antigens (Cheung *et al.*, 2006). CyaA* was also shown to act as a mucosal adjuvant when co-administered with other antigens by the intranasal route (Orr *et al.*, 2007). Moreover, since CyaA can deliver its N-terminal catalytic domain into antigen-presenting cells, and has several permissive sites that allows peptides to be inserted without altering the ability of the toxin to enter cells, recombinant CyaA toxoids have been designed to deliver epitopes into antigen-presenting cells to induce cell-mediated immune responses *in vivo* (Fayolle *et al.*, 2001).

1.4.1.2.3 Dermonecrotic toxin (DNT)

Dermonecrotic toxin (DNT), originally called heat-labile toxin, is a 102 kDa protein (Nakase and Endoh, 1985), whose activity is destroyed by heating at 60°C for 10 min. It induces lethality when injected intravenously or intraperitoneally into animals and inflammation, vasoconstriction and local dermonecrotic lesions when injected intradermally into mice (Endoh *et al.*, 1990). DNT is not secreted like the other *Bordetella* toxins and was therefore initially misidentified as an endotoxin (Bordet and Gengou, 1909).

The toxin is an A-B toxin composed of an N-terminal receptor-binding (B) domain and a C-terminal enzymatically active (A) domain (Matsuzawa *et al.*, 2002). Nearly identical proteins are produced by all three major *Bordetella* species (Locht, 1999) and by *B. avium*. DNT is another virulence factor that is controlled by the regulatory locus *bvg*. Avirulent phase *B. bronchiseptica* strains express some DNT but in much reduced amounts compared to wild-type strains (Pullinger *et al.*, 1996). It has also been shown that transposon mutants of *B. pertussis* lacking dermonecrotic toxin are not noticeably less virulent than wild type strains in mice (Weiss and Goodwin, 1989) and, therefore, its role as a virulence factor in pertussis remains obscure.

DNT is known to induce nuclear division in mammalian cells without subsequent cell division, resulting in bi- or multi-nucleation and stimulation of stress fibre formation. DNT binds to cells through its N-terminal region consisting of 54 amino acids (Matsuzawa *et al.*, 2002). This region appears to recognize and bind to a cell surface receptor specific to toxin-sensitive cells and not to resistant cells.

It has been reported that DNT inhibits the elevation of alkaline phosphatase activity and reduces the expression of type-I collagen in an osteoblast-like cell line (Horiguchi *et al.*, 1991). It also induces the assembly of actin stress fibres and tyrosine phosphorylation of focal adhesion kinase (Horiguchi *et al.*, 1994). Deamidation of glutamine 63, which is essential for GTP hydrolysis by Rho (the small GTP-binding protein), involving residues 1136 to 1451 of DNT is believed to be the reason for the effects of DNT (Schmidt *et al.*, 1999). Deamidation of glutamine by DNT inhibits the GTPase activity of Rho and causes the Rho protein to be constitutively active. Rho GTPases are regulators of the actin cytoskeleton and act as molecular switches to trigger several intracellular signalling pathways (Madan Babu *et al.*, 2001).

1.4.1.2.4 Tracheal cytotoxin (TCT)

The characteristic paroxysmal cough (pertussis) caused by *B. pertussis* is likely to be attributed, in part at least, to the elaboration of the tracheal cytotoxin (TCT). TCT production is not controlled via the two components BvgA/S regulatory system (Locht, 1999). Purified and biologically-active TCT contains amino acids and amino sugar residues consistent with its proposed peptidoglycan origin and it was found to be a released fragment of *B. pertussis* peptidoglycan (Cookson *et al.*, 1989). Tracheal cytotoxin (TCT) was discovered by its ability to cause ciliostasis (restriction of the free movement of cilia) and effects the killing and extrusion of ciliated epithelial cells in hamster tracheal organ cultures and inhibition of DNA synthesis in hamster tracheal epithelial cell cultures (Goldman *et al.*, 1982).

TCT stimulates release of intracellular interleukin-1 (IL-1) and of nitric oxide (NO), the most likely triggers of TCT-mediated cytopathy (Heiss *et al.*, 1994). The TCT-dependent increase in NO is proposed to be the cause of respiratory cytopathology in the ciliated epithelial cells of the bronchial tract associated with the infection. The production of NO in the airway epithelium is induced synergistically by the action of TCT and *Bordetella* LPS (Flak and Goldman, 1999). TCT, even at very low concentrations, also impairs neutrophil functions and may thereby contribute to survival of bordetellae *in vivo* (Cundell *et al.*, 1994). TCT is also produced by at least some other *Bordetella* species, *B. pertussis*, *B. parapertussis*, *B. bronchiseptica* and *B. avium*, suggesting that it may be of general importance in bordetellosis. A promising new attenuated live *B. pertussis* vaccine strain was developed by the inactivation or removal of genes for PT, DNT and prevention of release of TCT. The latter was accomplished by replacement of the *B. pertussis ampG* gene by *E. coli*

ampG, more efficient at recycling the petidoglycan fragments, resulting in expression of <1% of the original TCT activity. This strain was able to colonize the respiratory tract of mice and induce strong protective immunity after a single nasal administration (Mielcarek *et al.*, 2006).

1.4.1.2.5 Lipopolysaccharide

The outer membranes of gram-negative bacteria (Fig. 1.6) (such as *B. pertussis*) are composed of lipopolysaccharides (LPS), proteins and phospholipids that are organised in a complex manner. The outer membrane acts as a barrier to exclude harmful substances and explains the fact that Gram-negative bacteria are less affected than Gram-positive bacteria by lysozyme and penicillin, as well as by other antibiotics (Voet & Voet, 1995).

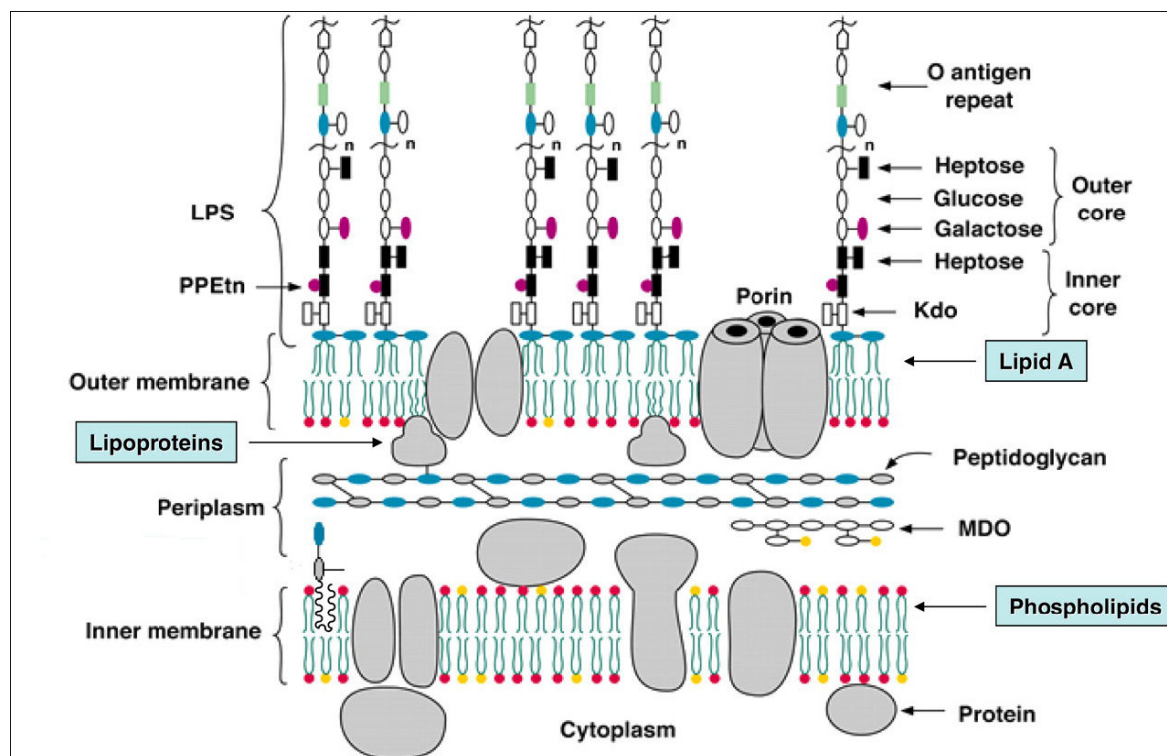


Figure 1.6 Schematic representation of the inner and outer membranes of *E. coli* K-12. Coloured ovals and rectangles represent sugar residues, whereas circles represent polar headgroups of various lipids (Raetz and Whitfield, 2002). Abbreviations: MDO, membrane-derived oligosaccharide; PPEtn, phosphoethanolamine; Kdo, 3-deoxy-d-manno-oct-2-ulonic acid; LPS, lipopolysaccharide

Introduction

LPS of Gram-negative bacteria, also called endotoxin, are amphipathic molecules and located in bacterial outer membranes (Figure 1.6). All LPS contain a non-polar lipid A, which anchors LPS in the outer membrane and is covalently attached to a heteropolysaccharide chain. This chain is composed of a core oligosaccharide and in some cases, an O-antigen. The heteropolysaccharide can contribute to the serological identity of the bacterium since it is exposed to the extracellular medium. The O-antigen is composed of repeating oligosaccharide units, which are specifically exhibited on each bacterial strain. The O-antigen confers smoothness to the organism, which decreases the likelihood that a bacterial cell will be phagocytised. The surface hydrophobicity of the organism is reduced by O-antigen to allow better interaction with other protein-coated membranes.

The LPS molecules from the three *bordetellae* (*B. pertussis*, *B. parapertussis* and *B. bronchiseptica*) share basic structural features such as the lipid A domain and a branched-chain core oligosaccharide (Lebbar *et al.*, 1994), but there are also considerable differences. One of the most important of these is that *B. bronchiseptica* and *B. parapertussis* synthesize a long chain polysaccharide structure including a homopolymer of 2,3-dideoxy-2,3-diN-acetylgalactosaminuronic acid (2,3-di-NAcGalA), whereas *B. pertussis* does not (DiFabio *et al.*, 1992). The endotoxin of *B. pertussis* is referred to as lipo-oligosaccharide (LOS) (Brodeur *et al.*, 1993). This structural difference between the LPS molecules of the three main pathogenic *bordetellae* leads to quite different surface properties of the different species.

The full structure of the LPS (LOS) of *B. pertussis* (Fig. 1.7) has been identified (Caroff, 2000). It is resolved as two separate bands (A and B) on silver-stained SDS-PAGE. Monosaccharides of the *B. pertussis* core oligosaccharide (band B) (faster-migrating band) are composed of charged galactosaminuronic acid (GalNAc), glucuronic acid (GlcA) and glucosamine (GlcN), which are not commonly found as components of other LPS core molecules. The lipid A of LPS is covalently linked to a single ketodeoxyoctulosonic acid (Kdo) residue and then to a branched-chain oligosaccharide (Allen *et al.*, 1998b).

The *wlb* gene locus (formerly *bpl*) (Reeves *et al.*, 1996) in *B. pertussis* is well conserved among the *Bordetella* subspecies using sequence homology studies. This gene locus is required for LPS trisaccharide biosynthesis (Allen *et al.*, 1998a) and is composed of 12 genes, *wlbA* to *wlbL*. The first two open reading frames in the *wlb* locus point leftwards and encode WaaA (for the addition of Kdo) and WaaC (for the addition of the first heptose).

The stop codon of the *waaC* gene overlaps with the start codon of *waaA*, which suggests that the two enzymes are translated together.

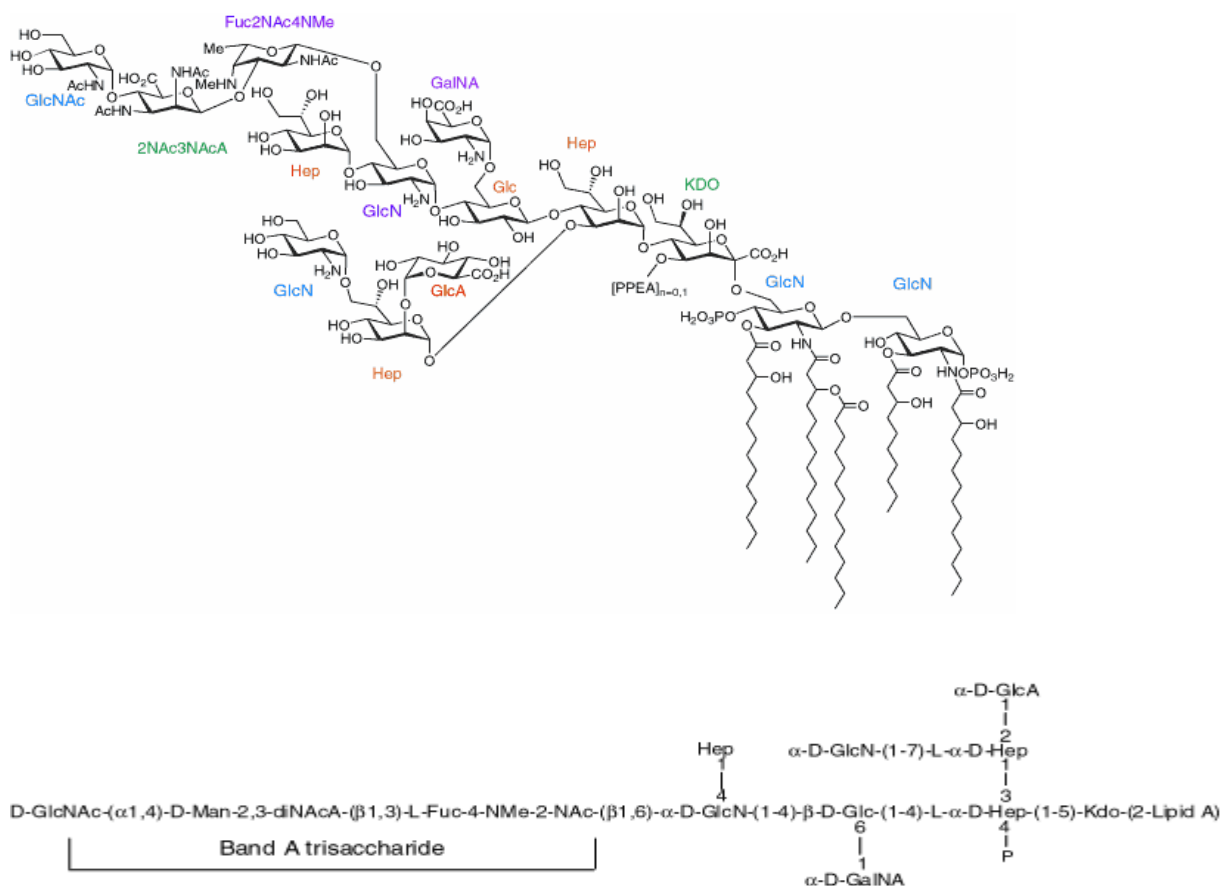


Figure 1.7 Chemical structure of *B. pertussis* LPS (LOS) and its abbreviated formula (From Caroff, 2000)

It has been suggested that *wlbA* encodes a dehydrogenase, *wlbB* encodes an acetylation enzyme, the product of *wlbC* transfers amino groups to sugars (and is homologous to WlbF), that of *wlbD* is a UDP-GlcNAc-2'-empimerase, which catalyses the formation of UDP-ManNAc, and *wlbE* encodes a sugar transferase. *wlbF*, *wlbG*, *wlbH*, encode a putative enzyme, a transferase, and a transferase, all of which are involved in adding sugars to the trisaccharide unit, respectively. *wlbI* encodes an integral membrane protein which is probably responsible for the export of band A and/or assembly of the final full-length LPS molecule. However, *wlbJ* and *wlbK* are two distinct genes in *B. pertussis*, but are in a single open reading frame in *B. bronchiseptica* and *B. parapertussis*. It has been shown that LPS biosynthesis is not affected by mutations in *wlbJK*. The *wbm* locus is located adjacent (immediately downstream) to the *wlb* locus in *B. bronchiseptica* and *B. parapertussis*, and is for the assembly of O antigen. The locus is replaced by an insertion sequence (IS) in *B.*

pertussis (Preston *et al.*, 1999). A deletion of *wbm* in these species led to loss of O antigen expression (Burns *et al.*, 2003). The Maskell group has constructed some *B. pertussis* mutants lacking the LPS band A trisaccharide, which have been shown to be severely compromised in their ability to infect mice (Maskell and Allen, 1996).

It has been suggested that changes of LPS structure in *B. bronchiseptica* is controlled by the BvgAS virulence regulatory system. A BvgAS-regulated lipid A palmitoyl transferase gene, *pagP*, is responsible for the palmitoylation of lipid A in *B. bronchiseptica* (Pilione *et al.*, 2004). However, identical open reading frames for *pagP* have been identified in *B. pertussis* and *B. paraptussis*, but the *pagP* gene is not expressed in *B. pertussis* due to a disruption of the putative promoter by an IS element (Pilione *et al.*, 2004).

LPS modification is a mechanism employed by Gram-negative bacteria during infection to resist host antimicrobial factors, avoidance of immune system recognition and maintaining a virulence phenotype (Gunn, 2001). These modifications include fatty acid additions, phosphoethanolamine (PEtN) addition to the core and lipid A regions, 4-amino-4-deoxy-L-arabinose (Ara4N) addition to the core and lipid A regions, acetylation of the O-antigen, and probably hydroxylation of fatty acids. Antimicrobial peptides (APs) are considered to kill micro-organisms by disturbing membrane integrity. These positively-charged peptides interact with negative charges in the LPS of Gram-negative bacteria (Gunn, 2001). It has been reported that LPS (LOS) protects *B. pertussis* from surfactant protein A (SP-A)-mediated clearance, by limiting access of SP-A to the lipid A region, the target for SP-A binding (Schaeffer *et al.*, 2004). LPS may also have a role in the resistance of *B. pertussis* to antibiotics (Turcotte, 1997). The lipid A pathway could therefore be an excellent target for the design of new antibiotics against Gram-negative bacteria, such as *Bordetella pertussis* (Onishi *et al.*, 1996).

It has been shown that the *B. bronchiseptica* is more resistant to complement than *B. pertussis* (Byrd *et al.*, 1991), and that BrkA does not mediate serum resistance in *B. bronchiseptica* (Rambow *et al.*, 1998). This difference could be explained by the compositions of their LPS. As explained above, in contrast to *B. bronchiseptica*, which produces long LPS molecules with many O-chain repeats similar to the LPS of *Escherichia coli*, *B. pertussis* has an abbreviated LPS structure and its O chain is composed of a single trisaccharide that is not repeated (Allen and Maskell, 1996). These long LPS molecules of the enterics are thought to confer resistance to complement by blocking the membrane attack complex from binding to the bacterial membrane (Moffitt and Frank, 1994). An O-

chain repeats deficient mutant of *B. bronchiseptica* was shown to be extremely sensitive to complement (Byrd *et al.*, 1991). However, the resistance of the avirulent-phase *bvg* mutant RB54 remains to be determined (Rambow *et al.*, 1998)

B. pertussis LOS has the properties of toxicity, pyrogenicity and adjuvanticity characteristic of endotoxin (Parton, 2004). It also has the ability to induce antiviral activity, B-cell mitogenicity and polyclonal B-cell activation (Chaby and Caroff, 1988). LOS in the whole-cell pertussis vaccine is considered to be non-protective and can contribute to the vaccine's toxicity and reactogenicity, therefore it was suggested that LOS should be excluded from acellular pertussis vaccines (Robinson *et al.*, 1985). However, *B. pertussis* LOS may contribute to induction of immunity. LOS signalling through toll-like receptor 4 (TLR4) in innate immune cells has been shown to play a critical role in the production of inflammatory cytokines such as IL-12, IL-23 and IL-1, which induce Th1 and Th-17 cells, after immunisation of mice with whole-pertussis vaccine, to finally promote bacterial killing by macrophages (Higgins *et al.*, 2006).

1.4.2 Other potential virulence factors

1.4.2.1 Outer-membrane protein P

B. pertussis produces a 40 kDa porin protein which complexes in the outer membrane to form a channel for anionic molecules and this porin resembles porin protein P of *Pseudomonas aeruginosa* (Armstrong *et al.*, 1986). The structural gene for this major porin, *ompP* was identified and sequenced by Li *et al.* (1991b). OmpP is expressed by Bvg⁺ and Bvg⁻ *B. pertussis* strains and Southern blot analysis also revealed the presence of the gene in *B. parapertussis* and *B. bronchiseptica*. In Bvg⁺ strains of *B. pertussis* this porin has the property of existing in a native crystalline pattern whereas Bvg⁻ strains did not display this property (Kessel *et al.*, 1988).

1.4.2.2 Outer-membrane protein Q

The gene *ompQ* encodes a porin-like protein OmpQ, which has a molecular mass of 39,133 Da (Finn *et al.*, 1995). A mutant of *B. pertussis* strain 18323 lacking OmpQ was not adversely affected in its ability to survive in the mouse aerosol challenge model and was not affected in its ability to survive *in vitro* (Finn *et al.*, 1995). The lack of effect was unexpected as Finn *et al.* (1995) had suggested that OmpQ may have an important role in

allowing the bacterium to have access to essential nutrients. In the mouse model system, it may be that other outer membrane proteins are able to substitute for OmpQ or the protein may not be involved in the infectious process. OmpQ is expressed along with other Bvg⁺ proteins and for this reason it was suggested that the protein plays a role during the infectious process in the human host or in the establishment of a carrier state. *ompQ* was present in *B. parapertussis* and *B. bronchiseptica* but was not found to be present in *B. avium* (Finn *et al.*, 1995).

1.4.2.3 *Bordetella* intermediate phase protein

Bordetella intermediate phase protein (BipA), a surface-expressed protein, is encoded by the gene *bipA*. BipA is expressed by *B. pertussis*, ovine *B. parapertussis* and *B. bronchiseptica* strains but not by human *B. parapertussis* strains (Fuchslocher *et al.*, 2003). RT-PCR analysis indicated that *bipA* was expressed maximally under semi-modulating (Bvgⁱ phase) conditions and that expression was controlled at the level of transcription (Stockbauer *et al.*, 2001). The C-terminus of BipA is exposed on the surface of *B. bronchiseptica* and, consistent with this topology, the predicted amino acid sequence of the N-terminal 500 amino acids shares significant similarity with the membrane localisation domains of EHEC and EPEC intimin and *Yersinia* invasin. The fact that *bipA* is absent from some strains and is not expressed or expressed at very low levels in others suggests that the role that BipA plays in the infectious cycle is either not essential or can be compensated for by other genes (Fuchslocher *et al.*, 2003). However, BipA could play some important role that is unique to the strains that express it.

1.4.2.4 *Vir90*

A potential virulence factor of *B. pertussis*, Vir90, has been described recently (Passerini de Rossi *et al.*, 2003). The protein sequence displayed closest homology with a number of ferrisiderophore receptors from Gram-negative bacteria. *vir90* open reading frames were also found in *B. parapertussis* and *B. bronchiseptica* genomes. This protein is encoded by a gene of 2322 bp, which is highly conserved between *B. pertussis* and *B. bronchiseptica* (99% homology) and between *B. pertussis* and *B. parapertussis* (98% homology). Its expression is under control of a vegetative promoter whose transcription could be environmentally-modulated, during infection, by the concerted action of BvgA and the repressor Fur (Passerini de Rossi *et al.*, 2003). It was suggested that Vir90 may play a role in the uptake of iron.

1.4.2.5 Subtilisin-like Ser protease/lipoprotein (SphB1)

Maturation of many secreted proteins in eukaryotes is performed by pro-protein convertases that belong to the superfamily of subtilisin-like proteases (Bergeron *et al.*, 2000). SphB1 is the first reported autotransporter whose α -domain provides a maturation factor for another protein secreted by the same organism. It has been identified as a subtilisin-like serine protease/lipoprotein, which is essential for the cleavage and maturation of FHA. As mentioned earlier (see section 1.4.1.1.2), the maturation and secretion of the 230 kDa FHA of *B. pertussis* requires the proteolytic removal of a ~130 kDa C-terminal intramolecular chaperone domain from the 367 kDa precursor (FhaB), which SphB1, a specific protease, is responsible for. SphB1 is probably not a general-purpose protease, and the maturation of FHA might be the major, if not only, function of it (Coutte *et al.*, 2001).

1.4.2.6 Virulence-activated gene-8 (*vag-8*)

B. pertussis expresses a *bvg*-regulated 95-kDa protein called Vag8, the fourth member of the *bordetella* autotransporter family to be identified (Finn and Amsbaugh, 1998). The N-terminal sequence of a 92-kDa protein described by Hamstra *et al.* (1995) identified this protein as Vag8 and a 91-kDa virulence-associated protein described by Armstrong and Parker (1986) can also now be identified as Vag8. The N-terminus of this polypeptide possesses a predicted 37 amino acid signal sequence and the cleavage of this would generate a mature protein of 91 kDa (Finn and Amsbaugh, 1998). Moreover, it is not cleaved away from its β -domain like some other *Bordetella* autotransporters. Southern blot analysis indicated that strains of *B. bronchiseptica* and *B. paraptussis* carry *vag8*-homologous DNA, yet expression of the protein has not been detected in *B. paraptussis* (Finn and Amsbaugh, 1998).

This protein showed greatest homology with the C-terminal ends of Prn, BrkA, and Tcf, with amino acid identity of their transport domains ranging from 33-44%. Vag-8 like other well-known *B. pertussis* autotransporters i.e. Prn, Tcf and BrkA, also contains an RGD site and two SGXG motifs (Fig 1.11). It has been shown that a Vag8-knock out derivative of *B. pertussis* colonized mice as efficiently as the parent *B. pertussis* strain in a mouse aerosol model of pertussis (Finn and Amsbaugh, 1998) so its role in virulence was unclear.

More recent data have indicated that Vag8 is involved in type III secretion in *B. bronchiseptica*. Genes encoding components of type III secretion system and its potential

translocated proteins have been identified in *B. bronchiseptica*, ovine *B. parapertussis*, human *B. parapertussis* and *B. pertussis*. Previously this secretion system appeared to only be expressed in *B. bronchiseptica* and ovine *B. parapertussis* strains (Yuk *et al.*, 1998; 2000) but it is now thought to be also expressed by *B. pertussis* and human *B. parapertussis* isolates (Hennuy *et al.*, 2002). *B. bronchiseptica* contains a *bvg*-regulated extracytoplasmic sigma factor (ECF), TrS, which controls the expression of virulence factors including the type III secretion system. Vag8 also seems to be regulated by TrS as analysis of the *vag8* promoter region revealed the presence of three putative sites where ECFs bind (Mattoo *et al.*, 2004).

1.4.2.7 Pertactin homologous gene (*phg*)

Phg is highly conserved in *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* but it is not present in *B. avium* and *B. petrii* (Middendorf *et al.*, 2005) and its role as a virulence factor is unclear. The ORF of this gene was named *phg* due to its relatedness to pertactin and it codes for a putative protein with 418 amino acids. The protein is integrated in the outer membrane, to form a β -barrel structure, where the N-terminal β -strand spans the outer membrane from the periplasmic space to the exterior (Middendorf *et al.*, 2005). It has been shown that the *phg* gene is continuously and slightly expressed in *B. pertussis* and *B. bronchiseptica*, those species that have been analysed so far. In Phg, the presumptive processing site is not conserved as compared to Prn, TcfA, BrkA and BapC (Middendorf *et al.*, 2005) and, the RGD adhesin motif is absent. In a virulence test of a kanamycin insertion mutant of Phg of *B. pertussis* in a mouse model of respiratory infection, it showed the same colonisation as the wild type strain, possibly because related proteins may compensate for the loss of its function.

1.4.3 Regulation of virulence genes

B. pertussis virulence factors are typically divided into two main categories: adhesins and toxins. Most are proteins, and the expression of their structural genes is coordinately regulated at the transcriptional level in response to environmental signals. *B. pertussis* can alternate between virulent and avirulent forms either by genotypic changes (serotype variation and phase variation) or by phenotypic changes (antigenic modulation) in response to growth conditions *in vitro* (Roberts and Parton, 2001).

Introduction

It has been shown that most of the virulence factors are not expressed at low temperature (except tracheal cytotoxin) or in the presence of MgSO_4 or nicotinic acid. This reversible phenomenon of adaptation in *B. pertussis* was named antigenic (phenotypic) modulation (Lacey, 1960). Among the population of *Bordetella* cells, irreversible loss of virulence factor production also occasionally occurs even at 37°C and in the absence of MgSO_4 and nicotinic acid, and has been termed phase variation, which indicates an irreversible alteration in the genotype caused by mutations. The genetic locus responsible for these phenomena is the *bvg* (*Bordetella* virulence gene) locus, composed of two closely linked genes, *bvgA* and *bvgS* which were identified as an example of the two-component family of transcriptional regulators. The *bvg* genes share homology with a family of prokaryotic regulatory proteins that react to environmental stimuli, and the *bvg* locus occupies ~ 5 kb on the total genome. BvgA, a transcriptional regulator, is a 23-kDa DNA-binding response regulator, and contains an N-terminal receiver domain and a C-terminal output domain with a helix-turn-helix (HTH) motif which is able to bind its DNA target sequences. BvgS is a 135-kDa transmembrane sensor kinase containing a periplasmic domain, a linker region (L), a transmitter (T), a receiver (R), and a histidine phosphotransfer domain (HPD) (Fig. 1.8). BvgS is located in the inner membrane of the organism and contains a large periplasmic domain, which is coupled to a cytoplasmic domain by a membrane spanning and a linker region. The BvgS is an unusual sensor among the two-component sensor proteins family since it has a particularly large cytoplasmic region with several subdomains (Locht, 1999). A proposed model suggests that signal inputs detected by the periplasmic domain are transmitted via the membrane to the transmitter (T) which autophosphorylates at His-729 (H) by a reaction that is reversible *in vitro*. His-729 then gives the phosphoryl group to Asp-1023 (D) of the receiver domain (R). Asp-1023 can provide the phosphoryl group to His-1172 of the HPD which can then relocate the phosphate to BvgA and thereby activate it.

BvgR, a 32-kDa cytoplasmic repressor and intermediate regulatory protein, is the product of the *bvgR* gene which is located directly downstream to the *bvgA* and *bvgS* genes (Merkel *et al.*, 1998). Its expression represses some of the genes controlled by the *bvgAS* operon. BvgA transcriptionally controls the expression of *bvgR*. While the BvgA/S system is activated, in the virulent phase, both virulence-activated genes (*vags*) (see below) and *bvgR* are expressed so as to in turn repress the virulence-repressed genes (*vrgs*). If BvgA/S system is inactivated, *vags* and *bvgR* are not expressed and *vrgs* are derepressed in modulating conditions such as at 25°C or in the presence of sulphate anion or nicotinic acid. This gives rise to an absence of *vag*-encoded factors and is termed the *Bvg⁻* phase or phenotype.

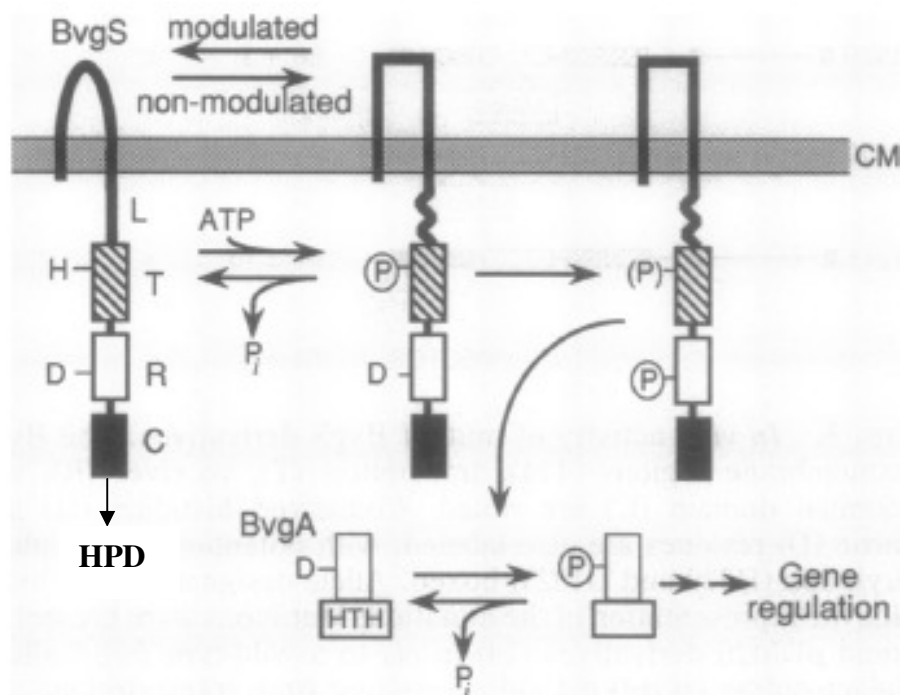


Figure 1.8 Model for the two-component signal transduction system by the BvgAS proteins (Uhl and Miller, 1994). Signal transduction by the BvgA/S system occurs

via a succession of phosphotransfer reaction through the transmitter (T), receiver (R), and C-terminal domain (C) of BvgS to the N-terminal receiver domain (D) of BvgA. Abbreviations: HTH, helix-turn-helix; HPD, histidine phosphotransfer domain; L, linker region; H, histidine

Transcription of the *bvgAS* locus is controlled by a 350 bp DNA fragment with four promoters. The transcription of the *bvg* locus is controlled by three of them, P₁, P₂ and P₃, whereas the fourth, P₄, is involved in the synthesis of an antisense RNA complementary to the RNAs synthesized by the P₁, P₂ and P₃ promoters (Fig. 1.9).

A low expression level of BvgA (the regulatory protein), which is inactive, is maintained under non-inducing conditions by the basally-active promoter P₂. BvgA is activated by phosphorylation, under inducing conditions, and it subsequently transactivates the promoters P₁, P₂, P₃, P₄, P_{FHA} and all other promoters of the *bvg* regulon or virulence-activated genes (*vags*). The *vags* form a group of loci including genes encoding the adhesins and toxins, activated through BvgA binding to *cis*-acting sequences in their promoter regions. This signifies the *Bvg*⁺ phase or phenotype. Hybridization of the antisense RNA coded from P₄ to the 5'-untranslated regions (20 bp upstream from the AUG start codon of the *bvgA* gene) of P₁, P₂ and P₃ positively regulates these promoters, since the activation of the P₄ promoter coincides with an increased production of BvgA (Scarlatto *et al.*, 1990). The *bvg* locus is also involved in the expression of a second class of *bvg*-regulated genes

during the Bvg⁻ phase, the virulence-repressed genes (*vrgs*) (Beattie, 1993). The products of these genes, such as *vrg6*, *vrg18*, *vrg24*, *vrg53* and *vrg73*, are much less well characterised than those of the *vags*.

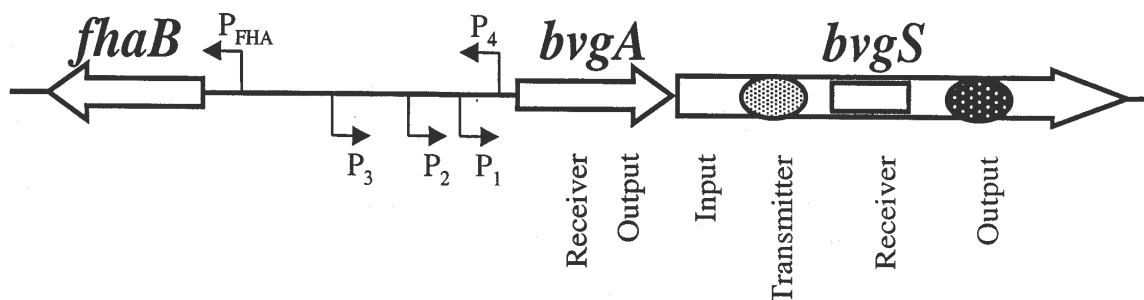


Figure 1.9 The *bvg* locus with *bvgAS* genes and the promoters P_1 , P_2 , P_3 and P_4 (Diagram from Madan Babu *et al.*, 2001). The role in signalling by the BvgA and BvgS proteins is indicated beneath the ORFs.

It is also important to note that BvgA can directly activate transcription of the *fha* and *bvg* promoters, while the *bvg* locus alone is not sufficient for the activation of the *ptx* and *cya* promoters. Apart from the BvgA, recent work has also confirmed the necessity of additional accessory factors such as the newly identified Baf protein or the effects of different DNA topology on the selection and activation of some promoters (Madan Babu *et al.*, 2001).

The BvgAS system controls expression of at least three distinct phenotypic phases (Bvg⁻, Bvgⁱ and Bvg⁺) by regulating the expression of at least four classes of genes (Williams and Cotter, 2006). Those that are expressed maximally only under Bvg⁺ phase (such as *cyaA*) are referred to as “late” Bvg-activated genes and require high concentration of Bvg~P which interact with primary binding sites with a relatively low affinity for Bvg~P (black curve in Fig. 1.10) (Beier and Gross, 2006). Those that are expressed maximally under both the Bvg⁺ and Bvgⁱ (intermediate) phases (such as *fhaB*) are characterised by high-affinity binding sites for Bvg~P and are referred to as “early” Bvg-activated genes (green curve in Fig. 1.10). Those that are expressed maximally only under Bvgⁱ phase conditions such as *bipA* are shown by the gold curve in Fig. 1.10. High-affinity binding sites for Bvg~P upstream of the RNA polymerase binding site are involved in *bipA* expression and low-affinity binding sites within the transcribed region counteract the activation of high-affinity binding sites and decrease the transcription at high Bvg~P concentrations (Williams *et al.*, 2005). Finally,

those that are repressed by BvgAS and expressed maximally only under Bvg⁻ phase conditions (such as genes involved in motility in *B. bronchiseptica*) are shown by the red curve in Fig. 1.10. Therefore, only Bvg-repressed genes are transcribed in the absence of Bvg~P (Beier and Gross, 2006).

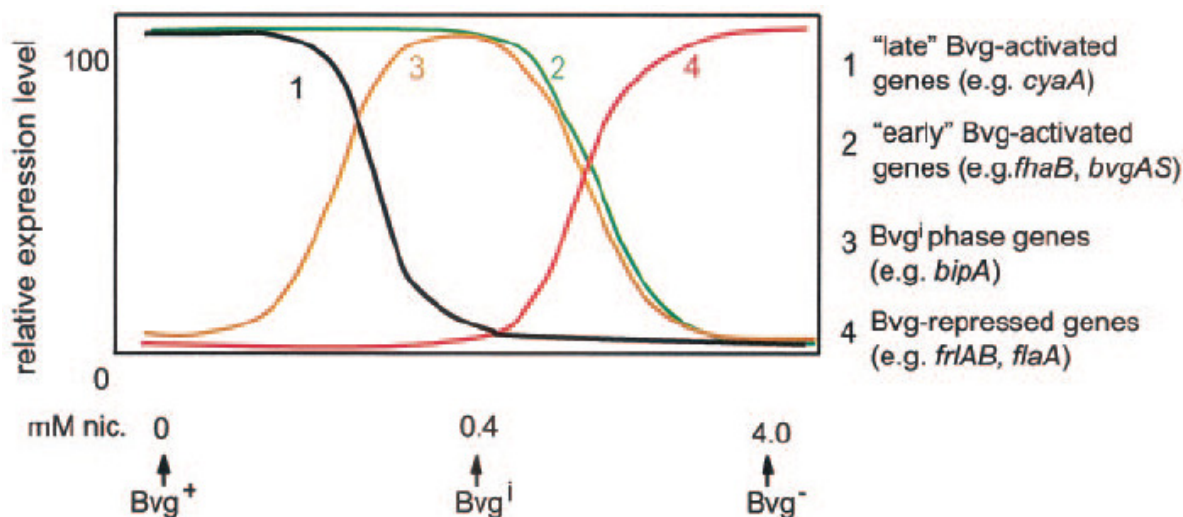


Figure 1.10 BvgAS controls expression of at least four classes of genes

(Adopted from Mattoo and Cherry, 2005). Systematic analysis of gene expression in the Bvg⁺, Bvgⁱ, and Bvg⁻ phases of *Bordetella* shows the existence of at least four classes of Bvg-regulated genes (see the text). Abbreviation: nic, nicotinic acid.

1.5 Other members of *B. pertussis* autotransporter family

The autotransporters are a growing family of extracellular proteins, found in many Gram-negative bacteria that have many different functions but appear to have the same mechanism of export (Henderson *et al.*, 1998). As the name implies, secretion through the autotransporter pathway does not require any accessory factors (See Type V secretion).

Recent work has shown that the *vag*-encoded virulence proteins in *B. pertussis*, namely: Tcf (Finn and Amsbaugh, 1998), BrkA (Fernandez and Weiss, 1994), pertactin (Charles *et al.*, 1989), Vag8 (Finn and Amsbaugh, 1998), and most recently Bap-5 or BapC belong to this family of proteins because of structural homology at their C termini (30-kDa). The N-termini also share structural characteristics: each contains one or more RGD and SGXG motifs (except pertactin), although the function of these proteins appears to differ. PRN (pertactin), BrkA, Tcf and Vag8 have already been described in previous sections and the

Figure 1.11 Structural features of some autotransporter proteins in *B. pertussis*

Table 1.1 *Bordetella* autotransporter proteins

Known or predicted <i>Bordetella</i> auto-transporter	ORF designation and no. of residues ^a			Accession no. ^b	Reference(s) or best BLAST hit of passenger	Bvg-regulation ^c	Known (predicted) signal peptide ^d	Notable motifs ^e
	Bp	Bpa	Bb					
Pertactin	BP1054, 910	BPP1150, 922	BB1366, 916	P14283	47, 61	+	34	RGD (2)
BrkA	BP3494, 1010	BPP0867, (173)	BB0961, (127)	AAA51646	132	+	42	RGD (2)
SphB1 ^f	BP0216, 1039	BPP0417, 1043	BB0419, 1039	CAC44081	82, 83	+	No	Serine protease lipoprotein
Tcf	BP1201, 647		BB3291, (145)	CAA08832	141	+	(39)	RGD
Vag8	BP2315, 915	BPP2415, 915	BB1864, 915	AAC31247	140	+	37	RGD
Phg	BP1767, 418	BPP1998, 415	BB2246, 415	CAB38010	None	0	(35)	None
SphB2	BP1660, 1024	BPP2745, (193)	BB2741, 1025		12	0	(37)	None
SphB3	BP1110, 1076	BPP2053, 1076	BB2301, 1076		12	0	(29)	Thiol protease
BapA (AidB) ^g	BP2224, 903	BPP2251, (295)[I]	BB1649, 903	CAC14165	AIDA-1	0	(30) ^h	None
BapB	BP1200, (119)[I]	BPP1815, 605	BB3292, 613	CAC14166	None	ND	(41)	None
BapC (BatE) ^g	BP2738, (102)	BPP2591, (100)	BB2033, 998	CAC14167	Pertactin	ND	(45)	RGD (2)
BatA	BP1793, (53)[I]	BPP2022, 538	BB2270, 536		None	ND	(33)	None
BatB	BP0529, 2300	BPP0452, 1769	BB0452, 2300		ShdA	ND	(38)	RGD (2), P-1 aspartyl protease
Novel	BP0775, 340	BPP0337, 340	BB0340, 340		None	ND	?	None
Novel	BP1344, 866	BPP2678, (717)	BB2830, 866		Ag43, AIDA-1, BapA	ND	(36)	RGD (2)
Novel	BP1610, (124)	BPP2975, 937	BB2941, 937		Pertactin	ND	32 ^h	None
Novel		BPP1617, 491	BB3111, 497		None	ND	(33)	None
Novel		BPP0449, 1616	BB0450, 2152		ShdA	ND	(40) ^h	P-loop, lipase
Novel		BPP0735, 996	BB0821, 999		YapE	ND	(30)	RGD
Novel		BPP0822, 1196	BB0916, 1196		SSP-h1	ND	(23) ^h	RGD
Novel		BPP1618, 519	BB3110, 705		Tcf	ND	BP1618 (33), BB3110 (54)	None
Novel	BP2627, (178)	BPP1256, 718	BB2324, 718				(34)	Lipoprotein

Adapted from Henderson *et al.* 2004.

others are described below. Figure 1.11 shows the major structural similarities among some of these autotransporter proteins of *B. pertussis*.

During the course of this current project, analysis of the genome in *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* has uncovered an inventory of 22 known or putative autotransporter proteins (Table 1.1) (Henderson *et al.*, 2004). In addition to those mentioned above, a-number of other autotransporters have been characterised to some extent. While most of the autotransporter ORFs are intact, not all appear to be expressed in all these species, due to frameshifts or insertion of a stop codon or insertion elements and these proteins are indicated in red. It is assumed that *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* have the capacity to express 12, 16, and 20 putative functional autotransporters, respectively (Henderson *et al.*, 2004). These proteins are indicated in blue. The nine putative autotransporters described in Table 1.1 as ‘novel’ include a putative adhesin and a putative lipase. SphB1 (subtilisin-like protease) (Coutte *et al.*, 2001) and Phg (Pertactin homologous gene) (Henderson *et al.*, 2004) have been described in earlier sections (1.4.2.5 and 1.4.2.7). However, SphB2, SphB3, BapA, BapB, BatA and BatB have been named and have homology with proteins/virulence factors in other organisms, are not yet fully-characterized. Some of these *Bordetella* autotransporter proteins are Bvg-regulated, some are not and some have not been yet determined. Table 1.1 also shows known or predicted signal peptides. Some of the autotransporters contain one or more RGD motifs (shown in green) (9 ATs) and several are predicted to have protease activity. The autotransporter that is the focus of interest in this thesis is described below.

1.5.1 *B. pertussis* autotransporter protein-5 (Bap-5) (BapC)

A PCR amplicon MR30 produced by Prof. M. Roberts (Department of Veterinary Pathology, University of Glasgow) using primers directed to the region encoding the C-terminal domain of pertactin on *B. pertussis* genomic DNA, was later identified as a gene for what was then the fifth member of *B. pertussis* autotransporter family (Blackburn, 1998). The gene sequence was deposited in GenBank under accession no. AF081494. In view of its sequence similarity to *prn* and genes encoding the three other proteins recognized as belonging to the autotransporter protein family (BrkA, Tcf and Vag8), the sequence was designated *bap-5* (Bordetella autotransporter protein 5). More recently, Henderson *et al.* (2000) reported the same sequence as *bapC*/BapC and this latter designation was used in the published *Bordetella* genome sequence (Parkhill *et al.*, 2003).

The presence of *bapC* in the genomes of *B. pertussis*, *B. bronchiseptica* and *B. parapertussis* has been identified via Southern blotting with a part of the MR30 amplicon in the *EcoRI*-digested genomes of these species (Blackburn, 2000). By using amino acid homology with Prn and BrkA, the ORF for BapC was predicted to begin at the serine residue encoded at nucleotide position 300, according to the sequence data of Blackburn (2000). This finding had suggested that a methionine upstream from this sequence (nucleotide position 261 according to the sequence data of Blackburn, 2000) might be the possible translational start. The presence of an upstream stop codon in this particular reading frame indicated that the open reading frame began at this point. Blackburn (2000), however, noted that there were alternative potential start sites and that it would require further study to ascertain the true ATG/GTG translational start codon. There were no translational stop codons within this open reading frame until position 2538 according to the sequence data of Blackburn (2000), which was consistent with previously characterized *B. pertussis* autotransporters as it is immediately preceded by a consensus outer-membrane localization motif.

Sequence analysis of the *bap-5* region by Blackburn (2000) did not reveal a typical Shine-Dalgarno sequence (Shine and Dalgarno, 1975) in the 5' region of its proposed open reading frame, as has been shown for other known autotransporter genes. It was also suggested that BapC, unlike other *B. pertussis* autotransporter proteins such as PRN, does not possess a typical amino-terminal signal sequence upstream from its putative translational start site (Blackburn, 2000).

Further study on the genome sequence has showed the existence of two potential BvgA dimer binding sites TTTC (A/G) TA upstream from the predicted translational start site of *bapC* (ATG1) according to the sequence data of Blackburn (2000). Upstream of the Bvg-regulated *prn*, *fha*, *ptx*, *cya* and *bvg* genes of *B. pertussis*, similar repeats are located. The BvgA dimer is required for regulating the transcription of these genes through its interaction with the RNA polymerase. No upstream regulatory elements (*e.g.* Ribosome binding site or promoter sequences) were identified in the *bapC* gene (Bokhari, 2002).

There appears to be a *rho*-independent terminator sequence downstream of the *bapC* gene as with *prn*, with the exception that it is further from the translational stop codon and smaller than that of *prn*. This potential transcriptional terminator was identified in the form of mRNA secondary hairpin structure, which is located downstream of the predicted ORF. This potential hairpin structure at the 3' end of the mRNA could be responsible for causing

the RNA polymerase to pause and therefore preventing further mRNA synthesis beyond the point of palindrome formation (Blackburn, 2000).

Sequence analysis of BapC, according to the sequence data of Blackburn (2000), showed the presence of an integrin-binding motif (RGD, position 945-953), a glycosaminoglycan-binding site (SGSG, position 1347-1358), a proteolytic processing site (ALSKRLGEL, position 1700-1727) and an outer-membrane localization signal (FHLGYRYRW, position 2510-2537) (Appendix II.3). The presence of an integrin-binding motif (RGD) within the N-terminus of BapC suggested that this protein may play a role in adhesion and invasion of the bacterium. Such motifs are present in the N-terminal domains of Prn, BrkA and Tcf. In Prn, the role of the RGD in adhesion and invasion has been studied using synthetic RGD-containing sequences in competitive binding experiments with HeLa cells. The result suggested that RGD in pertactin might play a role in adhesion and invasion (Leininger *et al.*, 1992). However, a RGD→RGE mutant of recombinant pertactin expressed by *E. coli* had no significant effect on adhesion to CHO or HepC cells (Everest *et al.*, 1996). The presence of a glycosaminoglycan-binding motif (serine-glycine-serine-glycine) (SGSG) provided further evidence that BapC may function as an adhesin and promote binding to glycosaminoglycans such as heparin. Such motifs are also present in BrkA and Tcf (Fig 1.11). However, as explained below, these conclusions were drawn from an incomplete knowledge of the structure of BapC.

The predicted molecular weight of BapC was 79.5 kDa using the first ATG translational start codon, according to the sequence data of Blackburn (2000). The processing of BapC at the putative cleavage site (ALSKRLGEL) would lead to a mature N-terminus product of 49.5 kDa and a 30-kDa β -barrel C-terminus domain in the outer membrane (Bokhari, 2002). However, during the course of the present investigation, with the publication of the genome sequences of three *Bordetella* species (Parkhill *et al.*, 2003), it became apparent that the *bap5/bapC* sequence identified by Blackburn *et al.* (1998) and Henderson *et al.* (2000) was not the whole *bapC* gene. As shown in Table 1.1, the ORF in *B. bronchiseptica* (strain RB50) encodes a protein of 998 amino acids. In *B. pertussis* Tohama and *B. parapertussis* (strain 12822), however, these ORFs were truncated at 102 and 100 amino acids, respectively, due to frameshifts. The *bap5/bapC* sequence in *B. pertussis* Taberman identified by Blackburn *et al.* (1998) and in strain Tohama by Henderson *et al.* (2000) showed 100% nucleotide identity with that of *B. pertussis* Tohama sequenced by the Sanger centre, although it was only the 3' end of the gene, downstream from the frameshifts. The non-mutated gene in *B. pertussis* was predicted to encode of protein of 993 amino acids

(reported by Sebaihia in 2006, GenBank Accession No. NP_888576), which would give a molecular weight of 104 kDa and would be processed, into a 74 kDa α -domain (including the signal sequence) and a 30 kDa β -domain.

Despite the above anomalies, the works of Blackburn (2000) and Bokhari (2002) had suggested that BapC was in fact expressed in the *B. pertussis* Taberman and Tohama strains that they used. Preliminary work on characterising the role of the BapC protein was undertaken by Bokhari (2002). The *bapC* gene amplified from *B. pertussis* strain Taberman was mutated by a kanamycin resistance cassette and incorporated into the suicide vector pSS1129 (Stibitz, 1989) for gene replacement into the chromosomes of *B. pertussis* strains Taberman and Tohama (Bokhari, 2002). Preliminary work, using an ELISA-based adhesion study, suggested that, with some cells such as HeLa 229, Hep-2 and murine macrophages RAW 264.7, the Taberman and Tohama *bapC* mutants adhered less well than the wild-type strain, and a rabbit anti-BapC serum had significant effect in reducing adherence of the wild-type strain. However, anti-BapC serum did not have an effect on adherence of the wild-type strain to other cells i.e. A549 (human lung epithelial cells), Caco-2 (human carcinoma intestinal cell line) and to P338D-1 macrophages, suggesting that the interaction between the bacterium and mammalian cells is complex and possibly depends on many different adhesins and receptors. Therefore, in those cases where the *bapC* mutant adhered less well than the wild-type strain or the anti-BapC serum reduced adherence, the interaction of BapC and its unspecified receptors may play a significant role in the adhesion process (Bokhari, 2002).

It was also suggested that BapC may have a role in *B. pertussis* Taberman and Tohama in resisting complement-mediated killing (Bokhari, 2002). BapC was shown to confer a considerable resistance on the *B. pertussis* Taberman and Tohama strains to killing by normal human serum compared to its BapC-deficient mutant. Moreover, it was shown that BapC protected *B. pertussis* apparently from the classical (antibody-dependent) pathway of complement activation. The sensitivity of the wild-type and mutant strains to normal human serum and to guinea pig complement alone was inhibited by the addition of EGTA.MgCl₂, suggesting that complement killing is Ca⁺⁺ dependent and therefore due to the classical pathway or MBL (Mannan Binding Lectin) pathway. However, the exact mechanism by which BapC mediates this protection was not investigated.

A mouse virulence test showed that BapC, like Prn, appeared to participate in establishing *B. pertussis* infection in the respiratory tract of the mouse. A *bapC* mutant of *B. pertussis*

strain Taberman had greatly reduced ability to persist and colonize the mouse lungs/trachea, when assessed at 7 days after intranasal administration. Mouse protection studies using the purified C-terminal domain of BapC suggested that it was not immunodominant or a protective region of the autotransporter protein (Blackburn, 2000), but no protection studies have been done with the whole BapC protein or the N-terminal moiety as the complete structure of BapC was not appreciated at the time.

The altered properties of the BapC mutants of *B. pertussis* strains Taberman and Tohama, compared with their wild types, provided evidence for BapC expression in these strains despite the fact that in the genome sequence of *B. pertussis* Tohama, *bapC* is a pseudogene (Table 1.1). Other evidence for expression of BapC in *B. pertussis* Taberman and Tohama was provided by RT-PCR and Western blotting. The RT-PCR and Western blotting results suggested that the expression of BapC is under *bvg*-regulation, like some other *B. pertussis* autotransporter proteins such as Prn and BrkA (Bokhari, 2002).

1.6 General mechanisms for secretion in Gram-negative bacteria

Pathogenic and non-pathogenic bacteria decorate their cell surfaces with, or secrete into the extracellular environment, many different proteins. The expression of virulence factors directly or indirectly affects the host and so increases the pathogen's ability to continue to exist and reproduce. In the case of Gram-positive organisms, which produce a single plasma membrane followed by a thick cell wall layer, surface proteins generally follow the *sec*-dependent pathway to cross the cell membrane. They are either released into the extracellular milieu or remain attached by one of several peptide-anchoring signals (Novick, 2000). Secretion in Gram-negative bacteria, however, is limited by the presence of the outer membrane, which requires more complex and specialized protein secretion pathways. At least five major protein secretion pathways (numbered from I to V), which are highly conserved, have been characterized for Gram-negative bacteria. They differ with respect to the mechanisms of outer membrane translocation but some have common inner membrane transport mechanisms.

1.6.1 Signal sequence-independent pathway (Type I):

The Type I *sec*-independent secretion system (T1SS) is exemplified by the secretion pathway defined for *Escherichia coli* haemolysin (HlyA) and other RTX toxins including *B.*

Introduction

pertussis CyaA (see section 1.4.1.2.2). Secretion requires three accessory proteins, which form a channel spanning both the inner and outer membranes (Stanley *et al.*, 1998), which allows substrate to secrete directly from the cytoplasm to the extracellular milieu (Fig 1.12). Three protein subunits consist of two cytoplasmic membrane proteins; the ABC transporter and membrane fusion protein (MFP), and an outer membrane protein (OMP) (Koronakis *et al.*, 2001). Type I secretion system transports various molecules, from inorganic ions, amino acids, sugars, peptides, to protein of various sizes (20 - 100 kDa). The membrane components required for secretion of HlyA from *E. coli* are shown in fig. 1.13.

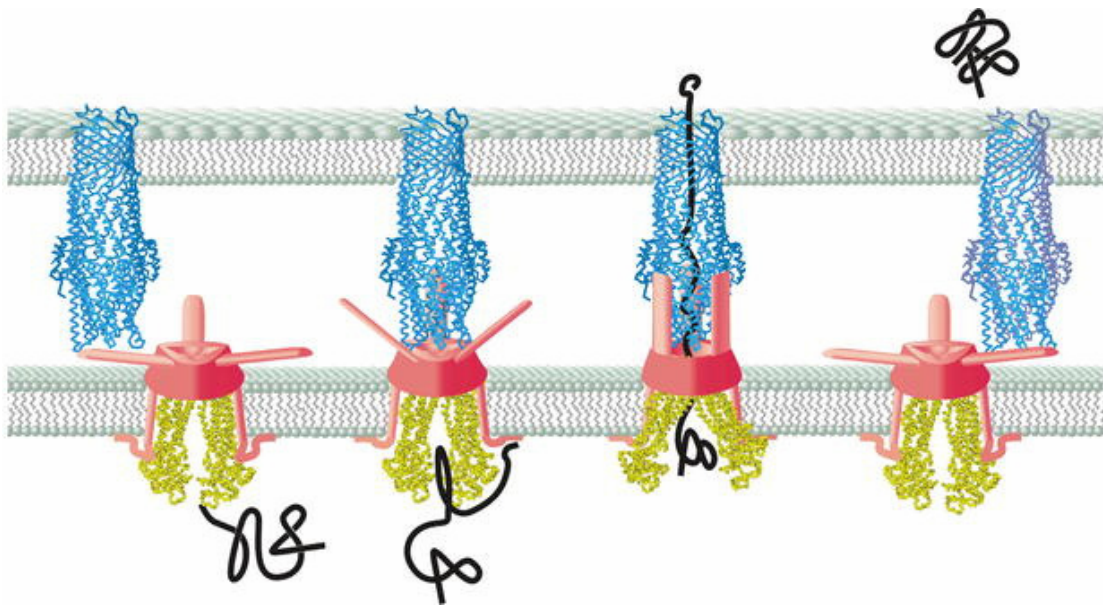


Figure 1.12 Model of the type I protein secretion system. A channel domain in the outer membrane anchors the channel-tunnel (blue). The membrane fusion protein (red) and the ABC (ATP-binding cassette) transporter (yellow) form a complex in the inner membrane. The preprotein (black line) binds to the transporter and the cytoplasmic part of the accessory membrane fusion protein (MFP) and induces a conformational change in the periplasmic domain of the accessory protein, allowing the recruitment of the channel-tunnel. A tight complex is formed, the tunnel entrance of the channel-tunnel is opened and the protein is secreted. After the process is finished the proteins return into their resting states (<http://archive.bmn.com/supp/ceb/ain1.html>).

Secretion of HlyA, and similar effector molecules, thus occurs in a Sec-independent manner and in a continuous process across both the inner and outer membranes. Moreover, proteins secreted by T1SSs are not processed during secretion and do not form separate periplasmic intermediates. HlyA is translocated simultaneously across the inner and outer membrane

(Fig. 1.13) in a reaction that requires ATP hydrolysis in the bacterial cytoplasm and interaction of trimeric HlyD with the trimeric outer membrane transporter TolC (pore-forming outer membrane protein).

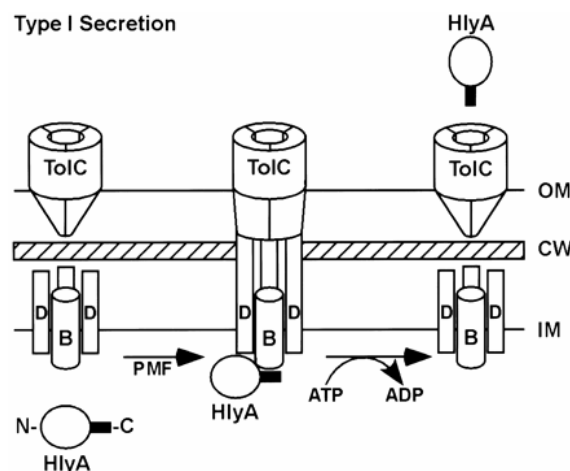


Figure 1.13 Type I secretion of RTX toxins. *E. coli* HlyA interacts with its associated ABC transporter (B = HlyB, ATP-binding cassette) and the trimeric membrane fusion protein (MFP) (D = HlyD), in a proton motive force (PMF) dependent manner. [Drawing adapted from Stanley et al., 1998]

1.6.2 General secretory pathway (GSP, Type II):

This protein secretion system is exemplified by the pullulanase (PulA) system of *Klebsiella oxytoca*. This type of secretion pathway is often referred to as the main terminal branch (MTB) of the Sec-dependent GSP. This system is able to secrete a large variety of proteins, which are synthesized as pre (pro) polypeptides. PulA secretion requires the action of approximately 14 additional accessory proteins, which are encoded in an operon (Pugsley, 1997). The type II system also features a macromolecular, multicomponent structure that most probably spans both inner and outer membranes (Nunn, 1999). Unlike the type I and type III secretion systems and some members of the type IV secretion system, secretion of pullulanase across the inner membrane is *sec*-dependent. This pathway is the transporter for many of the *B. pertussis* proteins like the autotransporter proteins pertactin, tracheal colonisation factor, as well as the major *B. pertussis* adhesion FHA and fimbriae across the inner membrane and into the periplasmic space. Type II secretion pathway is also presumed to be involved in transporting the pertussis toxin subunits into the periplasmic space.

Secretion of proteins through the MTB pathway is performed in a two-step Sec-dependent fashion. Proteins secreted via the type II pathway are translocated with an N-terminal signal sequence that targets proteins to the Sec inner membrane secretion pathway. The Sec machinery consists of an ATPase, SecA, several integral inner membrane proteins and a signal peptidase. The energy for transport through the translocon is provided by SecA through binding and hydrolyzing ATP, which leads to major conformational changes in this protein. During translocation signal peptidase cleaves the signal sequence and the remainder of the protein is released into the periplasm. It has been suggested that the protein acquires a native state in the periplasm, which is necessary for translocation across the outer membrane and performed by certain chaperones such as these involved in disulphide bond formation and isomerization (e.g. DsbA) or specific molecular chaperones. The translocation across the outer membrane, which is highly specific for the type II system, requires several additional accessory proteins (Sandkvist, 2001).

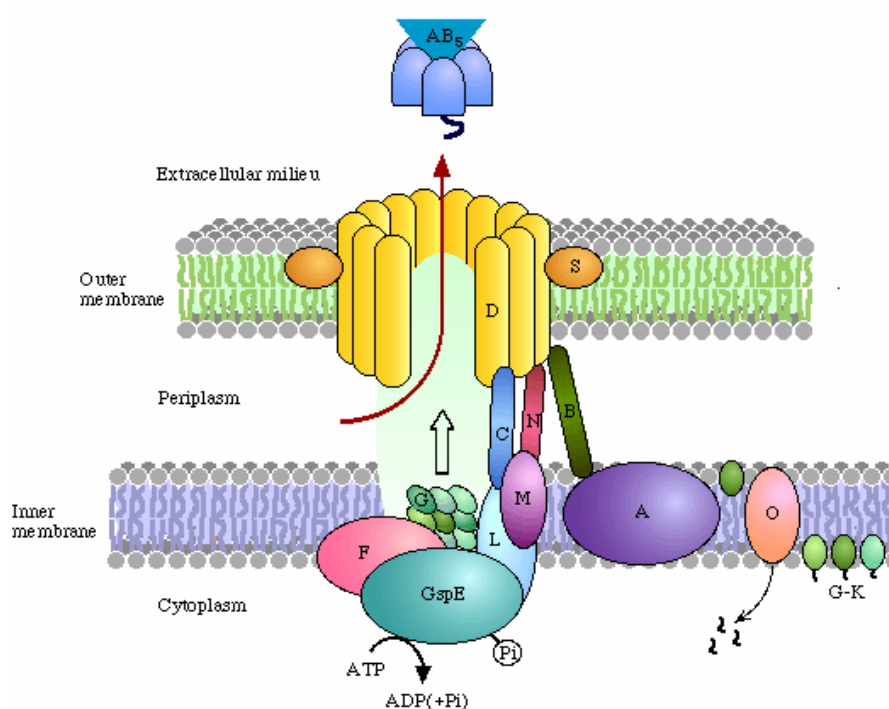


Figure 1.14 Type II secretion system of the pullulanase (PulA) in *Klebsiella oxytoca*. The secretion machinery can be depicted as comprising three building blocks: proteins GspE–GspF–GspL–GspM, which form a large platform in the inner membrane; the pseudopilus composed of GspG–GspH–GspI–GspJ–GspK proteins; and the gated outer membrane proteins GspC–GspD–GspS, which are able to form a pore in the outer membrane. (www.genome.ad.jp/kegg/pathway/map/map03090.html).

1.6.3 Type III secretion pathway (the injectisome):

The type III secretion system (T3SS) is a highly regulated process, first identified in pathogenic *Yersinia* spp, which allows Gram-negative pathogens to carry bacterial effector molecules into eukaryotic cells and change host cell signalling functions (Francis *et al.*, 2002). Although among type III secretion systems most of the secretion apparatus elements are conserved, the set of delivered effector proteins is often unique for each bacterial species. The ability to target effector molecules directly into eukaryotic cells is the most prominent feature of T3SS.

The type III secretion pathway has a primary role in the virulence of some pathogens such as *Salmonella enterica*, *Shigella flexneri*, *E. coli* and *Chlamydia trachomatis*. A complex machinery of proteins, which bring together into a strongly regulated oligomeric structure spanning the inner and outer membranes, is required for the secretion of type III effector molecules. Like the T1SS, the *sec* system is not required for secretion of the effector molecules; however the *sec* machinery is required for translocation of some of the structural component proteins of the secretion apparatus across the inner membrane (Galan and Collmer, 1999). Secretion of effector molecules may occur without the formation of periplasmic intermediates, and is through a needle-like structure which consists of 20 different proteins. This needle-like structure crosses both the inner and outer membrane, like a hypodermic needle. This phenomenon has been termed “contact dependent” (Zierler and Galan, 1995), since in some cases it is started when the bacterium contacts with a target cell, and the analogy of the T3SS with a hypodermic syringe to inject proteins directly into the host cytoplasm, has lead to the term “injectisome” to describe this secretion system.

The *Bordetella* type III secretion system has been studied in *B. bronchiseptica* (Stockbauer *et al.*, 2003), which appears to be the evolutionary progenitor for some *Bordetella* subspecies. A highly conserved type III secretion locus is shared by members of the *Bordetella* genus that cause respiratory infections in mammals (Yuk *et al.*, 1998). Type III secretion is positively-regulated in *B. bronchiseptica* at the transcriptional level by the products of the *bvgAS* locus (Cotter and Jones, 2003). A highly regulated region encoding the first 20 amino acids of the effector proteins is necessary for secretion and processing of these proteins via the type III pathway (Hueck, 1998).

The chromosomal *bsc* locus includes 22 genes that encode components of the *Bordetella* type III secretion apparatus, secreted proteins and putative chaperones and is similar to that

of *Yersinia* as shown in Fig. 1.15. In general, genes encoding components required for T3SS are located on a single plasmid or chromosomal locus, and are conserved among different species. In *B. bronchiseptica*, type III secreted polypeptides, which are easily detected in culture supernatants, are encoded by *bopB*, *bopD*, *bopN* and *bsp22* (Yuk *et al.*, 2000). BopB and BopD are orthologues of the *Yersinia* YopB and YopD translocation proteins (Cornelis, 2002). BopN is orthologous to YopN, which acts as a plug to avoid Yop secretion in *Yersinia* lacking host cell contact (Cheng *et al.*, 2001). DNA sequence analysis of the *Bordetella* genome in regions adjacent to the *bsc* locus has shown five well-conserved open reading frames (ORFs), *btrS*, *btrU*, *btrX*, *btrW* and *btrV*, which are activated by BvgAS and encode regulatory factors that control type III secretion at the levels of transcription, protein expression and secretion (Mattoo *et al.*, 2004).

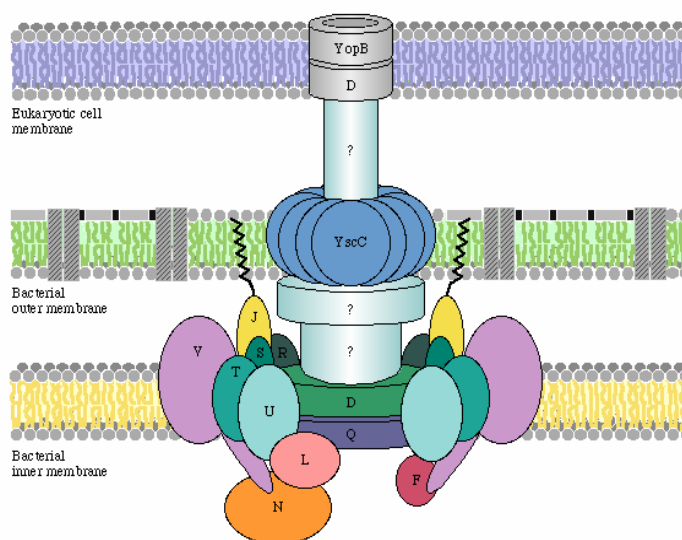


Figure 1.15 Type III secretion system in *Yersinia*. *Yersinia* spp exploits type III secretion for the delivery of effectors (YopE, YopH, YopM, YopN, YopO, YopP, and YopT) into host cells, begins with needle-forming machine components, providing for bacterial escape from phagocytic killing (www.genome.ad.jp/kegg/pathway/map/map03070.html).

1.6.4 Type IV secretion system (T4SS):

Type IV secretion systems are employed not only to carry macromolecules such as multimeric toxic molecules (such as pertussis toxin of *B. pertussis*, see section 1.4.1.2.1) or monomeric proteins to host cells, but also are utilized to transfer DNA or protein-DNA complexes from one bacterium to another (bacterial conjugation) or from a bacterium to a

eukaryotic cell (Vincent and Schneewind, 2001). Horizontal gene transfer, via conjugation systems, contributes to the evolution of infectious pathogens (Hacker *et al.*, 2003). Type IV secretion machineries play central roles in the spread of antibiotic resistance genes amongst bacteria by facilitating conjugative transfer. The IncF, IncP and IncW plasmid transfer systems (Tra) are some of the conjugal transfer systems (Lawely *et al.*, 2003). Type IV secretion systems are also involved in pathogenicity of bacteria, such as *Helicobacter pylori* responsible for peptic ulcers, or *Legionella pneumophila* responsible for Legionnaire's disease. Type IV secretion systems require a subclass of ATPases termed VirB11 ATPases (Fig. 1.16), which are crucial for conjugative transfer of DNA in most systems studied to date, and toxin transfer.

The role of VirB11 ATPase may differ from one organism to another relating to the type of transport they mediate such as in the conjugative system encoded by the RP4 plasmid, where the VirB11 ATPase, TrbB is identified to be involved in pilus biogenesis. Type IV secretion systems, particularly those involved in conjugative transfer; operate in conjunction with a fibrous cell surface organelle called a "pilus" which is considered to be essential for adhesion between bacteria or between bacteria and host eukaryotic cells. Nevertheless, there has not been an identified pilus associated with the type IV secretion system in *Helicobacter pylori* (the causative agent of peptic ulcers). Therefore, the VirB11 ATPase of *H. pylori* may be directly involved in transport of toxins through the inner membrane. It has been suggested that the potential roles of VirB11 proteins are at least in part associated with the inner membrane (IM) and localized to the cytoplasmic side of the IM.

The protein-DNA complex of *A. tumefaciens* is exported in a single continuous step from cytoplasm to the outside of the cell; however in the case of pertussis toxin, in a process resembling the type II secretion pathway, this occurs in two steps. The toxin subunits, PtxA and PtxB, are initially translocated across the cytoplasmic membrane via the Sec machinery and then targeted for export to the outer membrane. T4SS has been generally divided into two subclasses: type IVa correspond to protein machinery containing VirB homologues of *A. tumefaciens*, and type IVb corresponds to functional secretion systems assembled from Tra homologues of the IncI Collb-P9 plasmid of *Shigella flexneri* (Christie and Vogel, 2000).

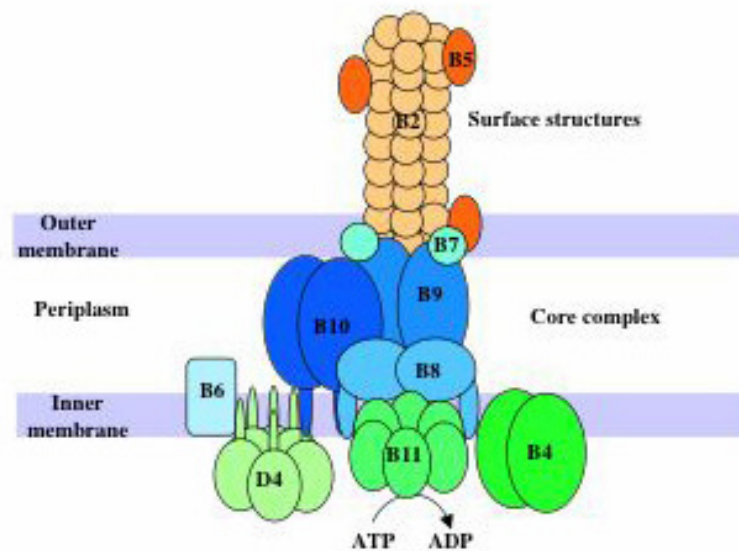


Figure 1.16 Type IV secretion system in *H. pylori*. Type IV secretion systems require a subclass of ATPases, VirB11 ATPases, which are essential for conjugative transfer of DNA in most systems, and toxin transfer (<http://people.cryst.bbk.ac.uk/~ubcg54.type4.htm>)

1.6.5 Autotransporters secretion mechanism (Type V secretion)

The autotransporter (AT) pathway is apparently the simplest process of secretion amongst the diverse pathways by which extracellular proteins can cross the outer membrane in Gram-negative bacteria. As their name implies, autotransporters do not require specific accessory molecules for secretion, and the information required for export is encoded inside the polypeptide itself. This family of secreted proteins includes those secreted via the autotransporter system (type Va or AT-1), the two-partner secretion pathway (type Vb), and the recently described type Vc system (also termed AT-2) (Desvaux *et al.*, 2004). The primary structures and modes of biogenesis of proteins secreted by these pathways are similar (Desvaux *et al.*, 2003) (Fig. 1.17). Various proteins including proteases, toxins, adhesins, invasins, S-layers and lipases, are exported to the cell surface via the autotransporter secretion system (Henderson *et al.*, 1998; Henderson and Nataro, 2001). These are typically virulence factors with different roles in pathogenesis (Kostakioti and Stathopoulos, 2004).

It has been shown that the genomes of the three *bordetellae* encode 22 autotransporters (Table 1.1). *B. bronchiseptica* encodes more autotransporters (20) than the other two *bordetellae* in terms of several of the genes encoding putative autotransporters are

pseudogenes or have been deleted from the genome of *B. pertussis* (13 autotransporters) or *B. parapertussis* (16 autotransporters) (Preston *et al.*, 2004).

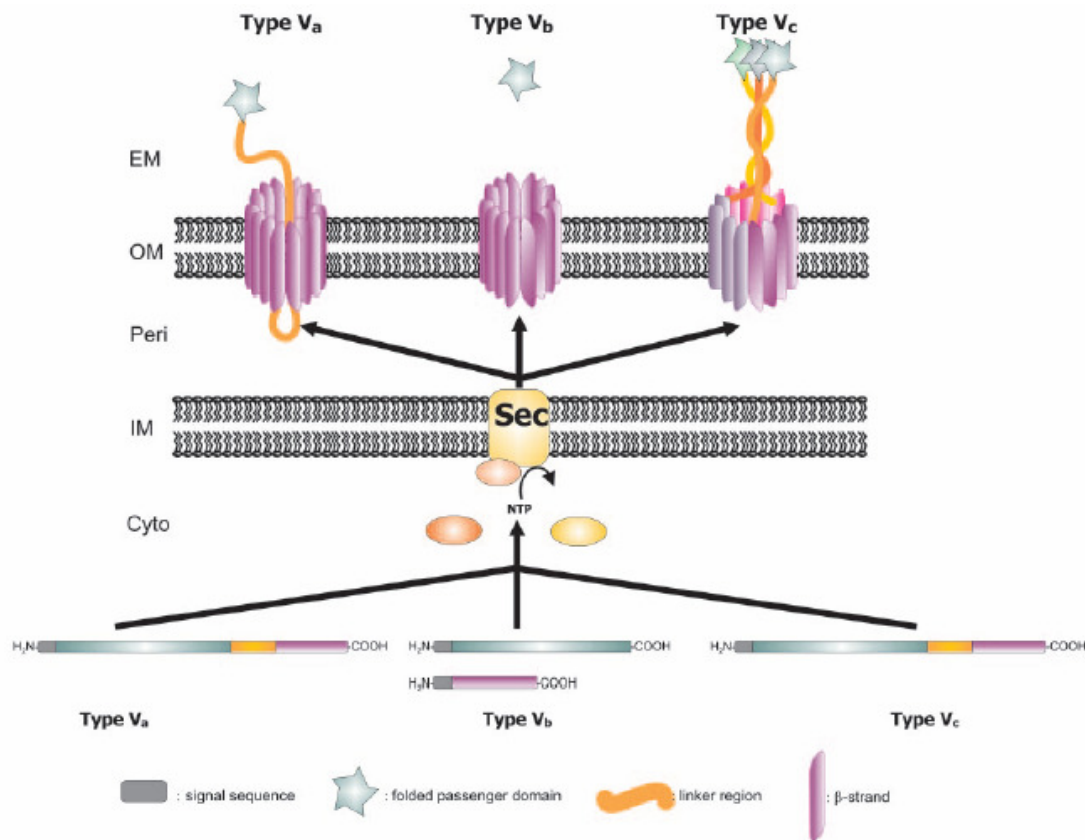


Figure 1.17 Schematic overview of the type V secretion systems. The signal sequence, the passenger (α) domain, the linker region, and the β -domain are the four functional domains of the proteins. The autotransporter polypeptides are synthesized and generally exported via the inner membrane using the Sec machinery. The signal sequence is then cleaved and the β -domain inserted into the outer membrane to form a β -barrel pore structure. The passenger α -domain is finally inserted into the pore and translocated to the bacterial cell surface, where it may or may not experience further processing (Henderson *et al.*, 2004).

1.6.5.1 Type Va (AT-1)

This secretion system was initially described for the immunoglobulin A1 (IgA1) protease of *Neisseria gonorrhoeae*, although many Gram-negative bacteria are now known to use this pathway to secrete a diversity of cell-surface and extra cellular enzymes and adhesins (Fig 1.18).

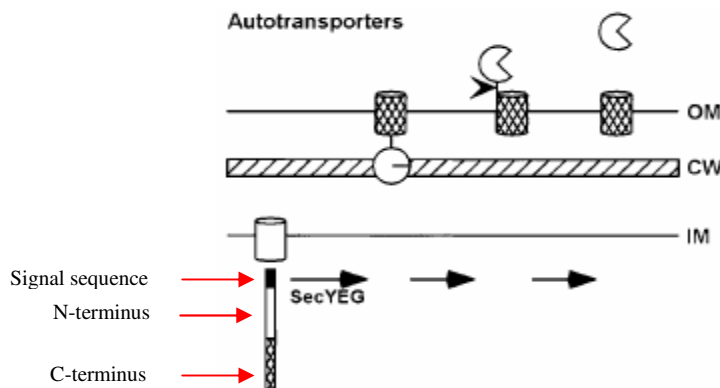


Figure 1.18 Type Va autotransporters. *Neisseria* extracellular IgA protease is synthesized as a pre-proenzyme. After Sec-mediated translocation across the inner-membrane, the C-terminal domain of the proenzyme inserts into the outer-membrane, which provides a pore to allow the N-terminal domain to translocate through its lumen. Protease activity can cleave the N-terminal domain from the C-terminal domain (arrowhead) and it is then released into the extracellular milieu. [Drawing adapted from Pohlner *et al.*, 1987]. SecYEG are components of the inner membrane-embedded translocation machinery.

Autotransporters are basically composed of three domains: a signal peptide (also called the signal sequence or leader sequence), the extracellular domain (also called the α -domain, N-passenger domain, or N-domain) and a translocation unit (also called the β -domain, helper domain, C-domain, transporter domain, or autotransporter domain) with characteristics comparable to those found in most outer membrane proteins. Translocation is performed in two steps including: (i) insertion of the C-terminal translocation unit into the outer membrane; and (ii) translocation of the passenger (Klauser *et al.*, 1992).

The passenger domain or α -domain confers the different effector functions of the various autotransporters. The C-terminus of these proteins (250 to 300 amino acid residues), the β -domain, is conserved and is necessary for the secretion of passenger proteins across the outer membrane (Yanagida *et al.*, 1986; Fischer *et al.*, 2001) by acquiring a tertiary structure once implanted into the outer membrane. In a model of autotransporter secretion (Henderson *et al.*, 1998), an N-terminal signal sequence facilitates translocation of the preproprotein across the inner membrane, via the Sec pathway. At the N-terminus of the protein, a Sec-dependent signal sequence allows targeting of the protein to the cytoplasmic membrane for its translocation into the periplasmic space. The signal sequence consists of (i) an n-domain containing positively charged amino acid residues, (ii) an h-domain

including hydrophobic amino acid residues, and (iii) a c-domain containing a consensus signal peptidase recognition site, which frequently has helix-breaking proline and glycine amino acid residues along with uncharged and short lateral-chain residues that define the site of signal sequence cleavage site (Martoglio and Dobberstein, 1998). Some proteins larger than 100 kDa possess extended signal sequences. It has been suggested that the unusually long signal sequence might serve to recruit accessory proteins (Henderson *et al.*, 1998). At least two pathways are employed in *E. coli* to translocate their proteins by the use of the Sec translocon: (i) molecular chaperone SecB, maintains the preprotein in a translocation-competent state and targets it to SecA (Randall and Hardy, 2002), and (ii) direct co-translational translocation of the preprotein by targeting the ribosome-SRP complex to the translocon (SRP pathway) (Herskovits *et al.*, 2000).

The autotransporters are produced in a pre-pro-protein structure in the bacterial cytoplasm and then released into the periplasm in a pro-protein form. The secretion of the autotransporters from the cytoplasm to the extracellular milieu is completed by the translocation through the outer membrane. The pro-protein, depending on the autotransporter, may then be cleaved from the β -barrel domain or remain coupled with the domain (Desvaux *et al.*, 2003). Protein (antifolding) chaperones either prevent the folding of the passenger domain to allow its translocation through the narrow channel of the β -translocator (upper panel of Fig 1.19) or translocate globular proteins with considerable tertiary structure, as in IgA protease β -barrel (lower panel of Fig 1.19). It was recently suggested that the IgA protease β -domain is capable of forming channels with an inner diameter of 2 nm (Veiga *et al.*, 2002). The channel possesses a 2.5–5 times smaller size than the other well-characterized type II and type III secretion system secretins that translocate folded proteins (Thanassi, 2002). However, this size of channel is sufficient for secreting small folded proteins or protein domains (Veiga *et al.*, 1999), although a channel of this size would be incapable of secreting larger folded passenger domains. These molecules must therefore be upheld in a translocation-competent unfolded state in the periplasm. It is not clear how such large proteins can retain an unfolded or partially folded state and not be degraded by periplasmic proteases. In this context, the formation of disulphide bonds in the passenger domain in the presence of DsbA (disulphide bond isomerase, chaperone) reduces the efficiency of secretion of the passenger domain which indicates the accessibility of the proprotein to periplasmic proteins (Brandon and Goldberg, 2001).

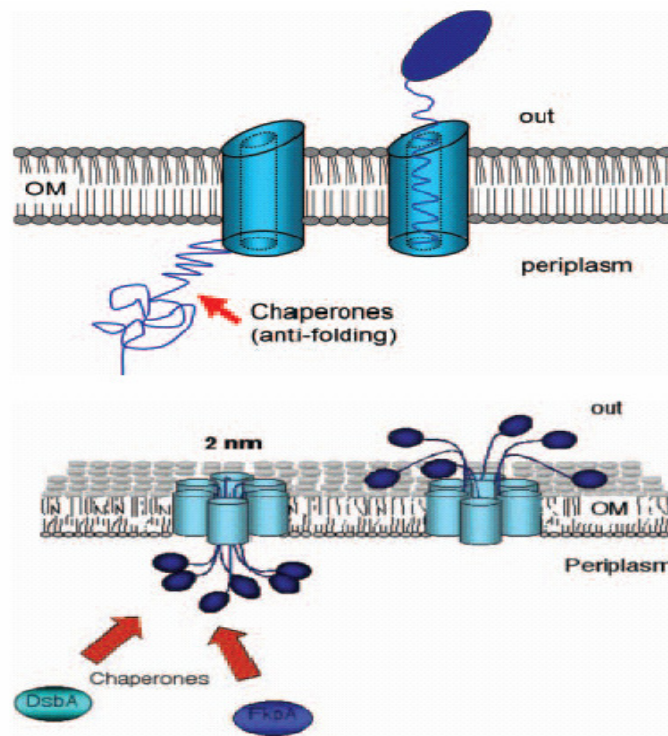


Figure 1.19 Model for outer-membrane translocation of passenger domains.

The passenger proteins are exported through a channel formed by a single β -barrel (upper panel) or multimers (lower panel). Folding of passenger proteins is either circumvented (upper panel) by periplasmic chaperones or supported (lower panel) before translocating across the outer membrane (Adapted from Pugsley *et al.*, 2004).

The crucial features necessary for passenger secretion are the β -core and a short preceding linker region (Miyazaki *et al.*, 1989; Oliver *et al.*, 2003a), together referred to as the translocation unit. Secondary structural analysis of autotransporter proteins (Klauser *et al.*, 1993; Maurer *et al.*, 1999; St Geme and Cutter, 2000) consistently predicts that the β -core forms an amphipathic β -barrel structure and the linker region forms α -structure (Henderson *et al.*, 1998; Oliver *et al.*, 2003b). This linker is an α -helical secondary structure in the N-terminus of the translocator domain placed on the periplasmic side of the outer membrane, folds into the aqueous pore, forming a hairpin structure that conducts the translocation of passengers through the channel (Oomen *et al.*, 2004). The passenger domain may finally be cleaved from the translocation unit and remain non-covalently connected to the bacterial surface, or released into the extracellular milieu. The cleavage of the passenger domain may happen by means of an autoproteolytic mechanism (if the passenger domain is a protease) or an endogenous outer membrane protease may mediate it (Oomen *et al.*, 2004).

Recently, the assembly and secretion of some outer membrane proteins has been shown to involve Omp85 (Fig. 1.20), which has homologues with highly conserved structure in all the complete genome sequences of Gram-negative bacteria. The Omp85 family has also been characterized in the outer membrane of mitochondria in eukaryotes from plants to humans and is an important element of the protein insertion machinery. However, the Omp85 was first shown to be involved in lipid export from the inner to the outer membrane. The Omp85 model hypothesizes that translocation of an AT involves Omp85, a protein that promotes the insertion of integral outer membrane proteins (Voulhoux *et al.*, 2003). It has been reported that, following Omp85 depletion in bacteria, unassembled forms of different outer membrane proteins including autotransporters were accumulated (Henderson *et al.*, 2004). Members of the Omp85 family of proteins are identified by the existence of two domains: (i) an N-terminal periplasmic domain, which is rich in POTRA (Polypeptide-Transport-Associated) repeats, and (ii) a C-terminal β -barrel domain inserted in the outer membrane. It has also been suggested that it has a chaperone-like function and interacts with the protein substrates prior to translocation by POTRA repeats (Gentle *et al.*, 2005).

It has been mentioned that lack of Omp85, a highly conserved protein, resulted in defective membrane insertion, folding and/or oligomerisation of many outer-membrane proteins (Robert *et al.*, 2006). It has also been shown that the secretion level of the proteolytically processed amino-terminal domains of BrkA expressed in an *E. coli* depletion background for Omp85 was reduced compared with the wild-type, suggesting that the *Bordetella* sp. are also dependent on Omp85 for proper outer membrane assembly (Jain and Goldberg, 2007). Moreover, the sequence similarity of Omp85 in Gram-negative bacteria with Toc75, which is involved in protein import into the chloroplast (Voulhoux and Tommassen, 2004), and the relationship with the two-partner secretion system of FhaC in *B. pertussis* (next section), suggests a common evolutionary origin (Gentle *et al.*, 2005).

Although the “Omp85 model” seems to be the most favoured (Oomen *et al.*, 2004) model for the insertion into and translocation across the outer membrane of passenger domains, several issues need to be addressed in future studies such as the role of Omp85 in the translocation of passenger domains if the passenger domain employs the translocation unit, and whether the translocation of passenger domain and the insertion of the translocation unit are two distinct steps or one single step (Rutherford and Mourez, 2006).

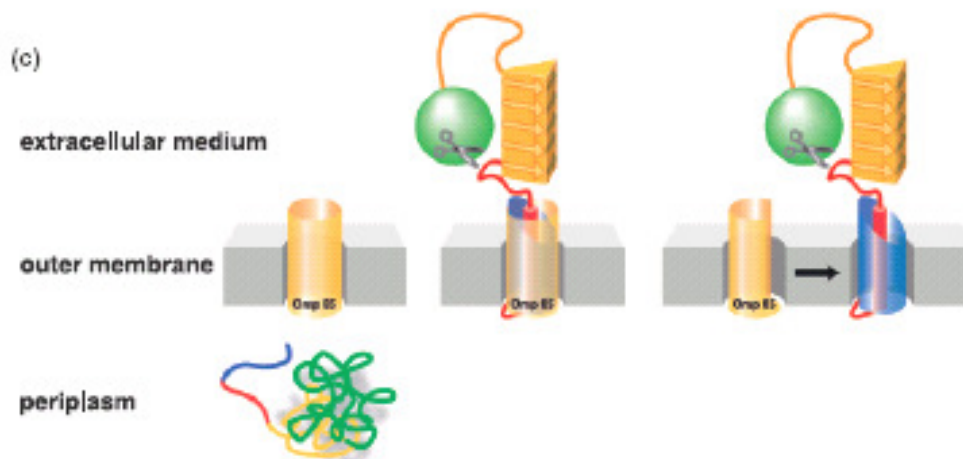


Figure 1.20 The translocator domain, because of its outer membrane protein characteristics, targets the Omp85 complex. The passenger is secreted via the Omp85 complex during insertion of the translocator domain into the outer membrane (Oomen *et al.*, 2004).

1.6.5.2 Two-Partner Secretion (TPS) Pathway (Type Vb):

TPS systems are distinct pathways, which share several similarities with the AT (type Va), and are composed of two separate proteins; TpsA the secreted protein (exoprotein) and TpsB the specific outer membrane transporter. The secreted proteins (passenger domain) (TpsA), like the autotransporter (AT) secretion pathway (Va), possess an N-terminal cleavable signal sequence, which directs translocation across the inner membrane in a Sec-dependent manner (Jacob-Dubuisson *et al.*, 2004). Some of the TpsA proteins have extended signal sequences of 40-70 residues, similar to some of the ATs, although the precise function of these extended signal peptides is unknown; their deletion did not inhibit secretion across the inner membrane (Lambert *et al.*, 1998). The TpsA is characterized by repeated amphipathic motifs predicted to adopt a β -helical structure (Kajava *et al.*, 2001). The TpsB proteins are large (approx. 60kDa), pore-forming outer-membrane transporters that are predicted to form β -barrels with 20-22 β -strands (Surana *et al.*, 2004). TpsB proteins are also synthesized with an N-terminal signal sequence for translocation across the inner membrane via the Sec system (Fig. 1.21). A family of very large proteins of over 1000 amino acid residues is secreted by using the two-step secretion mechanism. However, the

TPS systems are considered as autotransporters, but the passenger domain and the translocator domain are uncoupled and there is no sequence similarity between the TpsB transporter and the translocator domain of conventional autotransporters (Voulhoux *et al.*, 2003). The TpsB shows sequence similarity to Omp85 (see previous section).

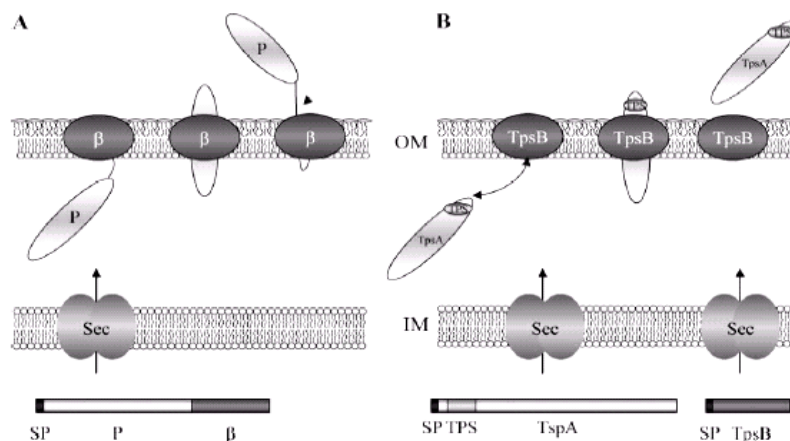


Figure 1.21 Comparison of the AT and TPS pathways. (A) The AT pathway. SP = signal peptide, P = passenger domain β = transporter domain. After export across the inner membrane via the Sec machinery, the translocation of the N-terminal passenger domain across the outer membrane is mediated by the β -domain, which embeds into the outer membrane. (B) The TPS pathway. TPS represents the TPS domain of the TpsA protein. After Sec-dependent translocation of both proteins, TpsB inserts into the outer membrane and then the TPS domain of the TpsA protein recognizes the TpsB transporter, which mediates the translocation of the TpsA across the outer membrane. The curved arrow symbolizes the specific interactions between the two partners, which seemingly occur in the periplasm (Jacob-Dubuisson, 2004). TpsA may then stay coupled with the cell surface or be released into the extra cellular milieu (Thanassi *et al.*, 2005).

Usually, the genes encoding the secreted protein (TpsA) and its cognate transporter (TpsB) are located in the same operon (Thanassi *et al.*, 2005). It is suggested that a TPS domain (Fig. 1.21), the hallmark of the TPS pathway with a 110-residue conserved region presumably interacts specifically with its cognate transporter to start translocation, which is essential for TpsA secretion or release into the extra cellular milieu (Fig. 1.21). It has also been suggested that the TPS domain may provide a recognition site for TpsB. *B. pertussis* FHA adhesin is one of the best-studied TPS systems (Jacob-Dubuisson *et al.*, 2004).

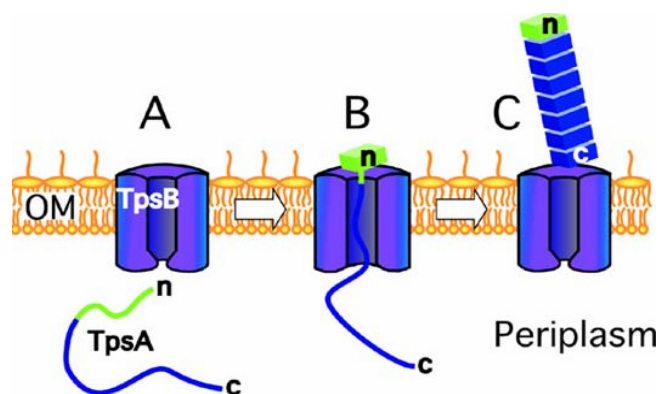


Figure 1.22 Model for secretion by the TPS pathway. Only secretion across the outer membrane is shown. (A) The N-terminal TPS domain (green) of TpsA binds to TpsB, possibly gating open the channel. TpsA secretion initiates from the N- to the C- terminus. (B) The TPS domain folds initially on the cell surface and then the TPS domain induces secretion and folding of the rest of TpsA into a repetitive β -sheet structure at the cell surface (C) (adapted from Thanassi *et al.*, 2005).

1.6.5.3 Type Vc (AT-2)

The AT-2 family has only been identified recently as a subfamily of surface-attached oligomeric autotransporters and consists of Hia of *H. influenzae*, the oligomeric coiled-coil adhesins (Oca) such as YadA of *Y. enterocolitica*, and some other proteins of α , β , and γ -Proteobacteria. However, the translocation unit of these proteins is much shorter than that of AT-1 proteins. The AT-2 family possesses all the aspects of ATs such as N-terminal signal sequence, a surface-exposed passenger domain, a linker region and a C-terminal domain predicted to form β -strands. It has been reported that the translocation unit of AT-2 appears to be a trimer consisted of 12 transmembrane β -strands (Oomen *et al.*, 2004) and is an alternative model for autotransporter secretion system (Cotter *et al.*, 2005) (Fig. 1.17).

1.6.6 Type VI and VII secretion systems

A novel system recently discovered in several Gram-negative pathogenic bacteria was named type VI (T6SS). T6SS gene clusters, which have been implicated in virulence-related processes by directly secreting virulence factors into host cells (Mougous *et al.*, 2006), are

highly conserved and are present in one or more copies in many pathogenic Gram-negative bacterial species such as *E. coli*, *P. aeruginosa*, *Y. pestis*, *V. cholerae* (Pukatzki *et al.*, 2007). The proteins secreted by this system lack a Sec signal peptide, but information on the identity, location and functions of most of the T6SS components remains unknown (Economou *et al.*, 2006). More recent evidence also showed a novel type VII secretion system, which mycobacteria employed to transport extracellular proteins across their hydrophobic and highly impermeable cell wall (Abdallah *et al.*, 2007).

1.7 Aims and Objectives

The main objective of the project was to characterize the BapC protein of *B. pertussis*. A suggested role in conferring serum resistance would be investigated, as well as the role of this protein in other virulence functions such as colonization of the mouse respiratory tract. As both BrkA and BapC are thought to contribute to serum resistance and mouse virulence, it was proposed to compare the role of BapC and BrkA in the same *B. pertussis* genetic background. The previous study (Bokhari, 2002) compared only mutants in different *B. pertussis* strains that may not have been otherwise isogenic. Also, by creating a *bapC*, *brkA* double mutant, it should be possible to determine whether it is fully serum sensitive or whether there are additional factors in *B. pertussis* that confer some degree of serum resistance. The authenticity of the mutants should be confirmed by PCR, Western blotting, Southern blotting, gene sequence analysis and slide agglutination tests.

The serum sensitivity and other properties of recombinant *E. coli* strains, which over-express the BapC, could be investigated and compared with the host strain. It was envisaged that the functions of BapC might be more easily studied in this strain than in *B. pertussis*, which expresses other autotransporter proteins with possibly similar or overlapping functions.

During the course of this investigation, it became apparent that not all *B. pertussis* strains or *Bordetella* species might express a functional BapC protein. Thus, some characterization of the *bapC* gene in different strains and species was undertaken.

2. Materials and Methods

2.1 General bacteriological procedures

2.1.1. *Bacterial strains, storage and growth media*

The details of *E. coli* strains used in this study are given in Table 2.1. *Bordetella* strains used in the study are given in the Table 2.2. Unless otherwise stated, all strains were available from culture collections within the Division of Infection and Immunity, University of Glasgow. The compositions of the following media used in the study are given in Appendix I.1. Luria Bertani (LB) broth; Luria Bertani agar; Bordet-Gengou (BG) agar; Casamino acids (CAA) solution; Cyclodextrin liquid (CL) medium; SOC medium; Stainer-Scholte (SS) and modified Stainer-Scholte media. All media were sterilised by autoclaving at 15 p.s.i (121°C) for 15 min unless stated. Heat-labile ingredients such as antibiotics (Table 2.3) and vitamin supplements were sterilised by filtration through a sterile 0.22 µm membrane filter (Gelman Sciences, USA) and were added to the growth media at appropriate concentrations. Glassware was sterilised by heating to 160°C for 2h.

For long term storage, the *E. coli* strains were kept at -70°C in LB broth supplemented with 50% (v/v) glycerol. *Bordetella* strains were resuspended in 1% CAA containing 20% (v/v) glycerol and stored at -70°C. Long term storage was performed by freeze drying to increase shelf life of *Bordetella* strains.

2.1.2. *Growth of B. pertussis strains*

B. pertussis was grown routinely on Bordet-Gengou (BG) agar containing 15% (v/v) defibrinated horse blood and 1% (v/v) glycerol. Plates were placed in a humidified environment (e.g. sandwich box containing a beaker of water) at 37°C for up to 3 days. The *B. pertussis* colonies can be maintained for a period of 7-10 days on BG agar plates stored inverted at room temperature or at 4°C in humidified conditions.

For liquid culture, *B. pertussis* was inoculated from BG plates into Stainer-Scholte (SS) liquid medium or Cyclodextrin Liquid (CL) medium and grown for 3 days at 37°C with shaking at 150-200 rpm. A loopful of bacteria was inoculated into 5 ml of liquid medium in

a universal or approximately 1.5 ml of bacterial culture was inoculated into 250 ml of liquid medium in a 500 ml flask.

To check the identity and purity of *B. pertussis* strains, Gram staining, plating onto nutrient agar and BG sandwich agar, for β -haemolysis, and slide agglutination tests with anti-*B. pertussis* serum were carried out. For preparation of the BG sandwich plates, a layer of BG agar without blood was poured onto a plate. Once set, a thin layer of BG agar with blood was added on top.

2.1.3. Growth of other *Bordetella* species

B. parapertussis, *B. bronchiseptica*, *B. avium*, *B. hinzii*, *B. holmesii*, and *B. trematum* were grown routinely at 37°C on BG agar plates for 48-72h. Where necessary, 500 ml of CL or SS medium in 2L dimpled flasks were inoculated from such BG plates and incubated for 48-72 h at 37°C with shaking at 150-200 rpm.

2.1.4. Growth of *E. coli*

E. coli strains were grown overnight on Luria-Bertani (LB) agar at 37°C. Where necessary, 500 ml of LB broth in a 2 L dimpled flask was inoculated from such plates or with 5 ml of liquid culture grown overnight in LB medium in a universal bottle. Then, the flasks were incubated overnight at 37°C with shaking at 150-200 rpm. Stocks of *E. coli* were prepared by adding 420 μ l of 70% (v/v) sterile glycerol to 580 μ l of an *E. coli* overnight culture in LB broth and stored at -80°C

Table 2.1 *E. coli* strains used in this study

Strains	Genotype/Phenotypes	Sources/Remarks
<i>E. coli</i> M15p (REP4)	Nal ^S , Str ^S , Rif ^S , Thi ⁻ , Ara ⁺ , Gal ⁺ , Mtl ⁻ , RecA ⁺ , Uvr ⁺ , Lon ⁺	Qiagen; Recombinant protein expression strain
<i>E. coli</i> M15pQE60 (ATG1)	<i>E. coli</i> M15p (REP4) above, also containing pQE60 (ATG1)	Blackburn PhD thesis, 2000
<i>E. coli</i> TOP10	F' { <i>lacI</i> ^q , <i>Tn10</i> (Tet ^r)}, <i>mcrA</i> , D(<i>mrr-hsdRMS-mcrBC</i>) <i>f80lacZDM15</i> <i>DlacX74</i> , <i>deoR</i> , <i>recA1</i> , <i>araD139</i> D(<i>ara-leu</i>)7697, <i>galU</i> , <i>galK</i> , <i>rpsL</i> (Str ^R), <i>endA1</i> , <i>nupG</i>	Invitrogen; General transformation strain
<i>E. coli</i> DH5 α	F'/ <i>endA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44 thi</i> ⁻¹ <i>recA1 gyrA</i> (Nal ^r) <i>relA1</i> Δ (<i>lacI</i> ZYA- <i>argF</i>)U169 <i>deoR</i> (F80 <i>dlacD(lacZ)</i> M15)	Invitrogen; General transformation strain
<i>E. coli</i> JM109	<i>recA1</i> , <i>supE44</i> , <i>endA1</i> , <i>hsdR17</i> , <i>gyrA96</i> , <i>relA1</i> , <i>thi</i> Δ (<i>lac-proAB</i>)	Promega; General transformation strain
<i>E. coli</i> SM10. λ _{pir}	<i>recA::RP4-2-Tc::Mu</i> , λ _{pir} , R6K, <i>thi</i> , <i>thr</i> , <i>leu</i> , <i>tonA</i> , <i>lacY</i> , <i>supE</i> ,	Division of Infection and Immunity, University of Glasgow; DNA mobilising strain
<i>E. coli</i> XL1 Blue	<i>recA1</i> , <i>endA1</i> , <i>gyrA96</i> , <i>thi-1</i> , <i>hsdR17</i> , <i>supE44</i> , <i>relA1</i> , <i>lac</i> [F' <i>proABlacIqZ</i> Δ M15 <i>Tn10</i> (Tetr)]	Stratagene; Host strain for routine cloning applications
<i>E. coli</i> BL21(DE3)*	F ⁻ <i>ompT gal dcm lon hsdS_B</i> (r _B ⁻ m _B ⁻) λ (DE3 [<i>lacI lacUV5-T7 gene 1 ind1 sam7 nin5</i>])	Invitrogen; Recombinant protein expression strain
<i>E. coli</i> BL21(DE3)pLysS**	F ⁻ <i>ompT gal dcm lon hsdS_B</i> (r _B ⁻ m _B ⁻) λ (DE3) pLysS(cm ^R)	Invitrogen; Recombinant protein expression strain

**E. coli* BL21(DE3) is ideal for use with bacteriophage T7 promoter-based expression systems (e.g. pET). BL21(DE3) carries the lambda DE3 lysogen. Recombinant proteins that are non-toxic to *E. coli* are generally expressed at higher levels in strain BL21(DE3) than in BL21(DE3)pLysS (see below). The basal expression levels of heterologous genes are significantly higher in BL21(DE3) than in BL21(DE3)pLysS.

***E. coli* BL21(DE3)pLysS is also used with T7 promoter-based expression systems (e.g. pET). This strain carries both the DE3 lysogen and the plasmid pLysS. pLysS constitutively expresses low levels of T7 lysozyme, which reduces basal expression of recombinant genes by inhibiting basal levels of T7 RNA polymerase.

Table 2.2 *Bordetella* strains used in this study

Species/Strains	Genotype/ Phenotype	Source
<i>B. pertussis</i> Taberman	Wild-type	Isolated from a child post-mortem. Ruchill Hospital, Glasgow (1979)
<i>B. pertussis</i> Taberman I	Sm ^r and Nal ^r derivative of BP Taberman parent	Division of Infection & Immunity, University of Glasgow (Bokhari, 2002, PhD Thesis)
<i>B. pertussis</i> Taberman II	BP Taberman I <i>bapC</i> ::Kan; Nal ^r , Sm ^r , Kan ^r	Division of Infection & Immunity, University of Glasgow (Bokhari, 2002, PhD Thesis)
<i>B. pertussis</i> Tohama	Wild-type	Division of Infection & Immunity, University of Glasgow
<i>B. pertussis</i> Tohama I	Sm ^r and Nal ^r derivative of BP Tohama wild-type	Division of Infection & Immunity, University of Glasgow (Bokhari, 2002, PhD Thesis)
<i>B. pertussis</i> Tohama II	BP Tohama I <i>bapC</i> ::Kan; Nal ^r , Sm ^r , Kan ^r	Division of Infection & Immunity, University of Glasgow (Bokhari, 2002, PhD Thesis)
<i>B. pertussis</i> BP338	Derivative of BP Tohama (Nal ^r). Parent of BP2041 and BP347	Weiss, A. Dept. of Molecular Genetics, University of Cincinnati
<i>B. pertussis</i> BP2041 [BP338 (BrkA ⁻)]	BP338 <i>brkA1</i> ::Tn5 Nal ^r , Kan ^r	Weiss, A. Dept. of Molecular Genetics, University of Cincinnati
<i>B. pertussis</i> BP347 [BP347 (Bvg ⁻)]	BP338 <i>bvgS1</i> ::Tn5 Nal ^r , Kan ^r	Weiss, A. Dept. of Molecular Genetics, University of Cincinnati
<i>B. pertussis</i> 18323	Wild-type, standard mouse-virulent strain	Division of Infection & Immunity, University of Glasgow
<i>B. pertussis</i> PICU		Originally from HPA, Colindale
<i>B. parapertussis</i> NCTC5952	Wild-type	Division of Infection & Immunity, University of Glasgow
<i>B. bronchiseptica</i> 214	Wild-type	Division of Infection & Immunity, University of Glasgow
<i>B. avium</i> 4480	Wild-type	Division of Infection & Immunity, University of Glasgow
<i>B. hinzii</i>	Wild-type	Division of Infection & Immunity, University of Glasgow
<i>B. holmesii</i>	Wild-type	Division of Infection & Immunity, University of Glasgow
<i>B. trematum</i>	Wild-type	Division of Infection & Immunity, University of Glasgow
<i>B. pertussis</i> BP338S	Sm ^r derivative of BP338	This study
<i>B. pertussis</i> BP2041S	Sm ^r derivative of BP2041	This study
<i>B. pertussis</i> BP338 (BapC ⁻)	BP338S <i>bapC</i> ::Tet Nal ^r , Sm ^r , Tet ^r	This study
<i>B. pertussis</i> BP338 (BrkA ⁻ , BapC ⁻)	BPM2041S <i>bapC</i> ::Tet Nal ^r , Kan ^r , Sm ^r , Tet ^r	This study

Table 2.3 Antibiotics used in this study

Antibiotic	Final concentration (µg/ml)
Ampicillin (Ap)	100
Cephalexin	100
Chloramphenicol (Cm)	20 and 34
Gentamicin sulphate	40
Kanamycin (Km)	40
Nalidixic acid (mono-sodium salt) (Nal)	40
Streptomycin (Sm)	100
Tetracycline (Tet)	2-40

2.1.5. Spontaneous antibiotic-resistant strains of *B. pertussis*

It is often desirable, in the genetic manipulation of bacteria, to utilise strains that are easily selectable, for example on antibiotic-containing plates. Streptomycin and nalidixic acid are the most commonly used antibiotics for generating spontaneous antibiotic-resistant strains of *B. pertussis*. In order to select such strains, a 10 ml volume of a mid-log phase culture was centrifuged and resuspended in 0.1-0.2 ml of fresh Stainer-Scholte medium. Aliquots (100 µl or 50µl) of bacterial suspension were then spread on BG agar plates containing 100 µg/ml of streptomycin or 40µg/ml of nalidixic acid and incubated at 37° for 4-5 days in humidified conditions to allow the growth of spontaneous resistant strains.

2.2 DNA extraction

2.2.1 Isolation of genomic DNA

For routine isolation of genomic DNA from *B. pertussis*, Promega's Wizard® genomic DNA purification kit was used. Bacteria from 1ml volume of an overnight culture grown, in appropriate medium, to OD_{600nm} ~ 0.6-0.7 were harvested by centrifugation at 13000 rpm using a tabletop micro centrifuge (Biofuge, Heraeus, Germany) for 2 min in a 1.5 ml micro centrifuge tube, and the supernate was discarded. 600 µl of Nuclei Lysis Solution (Promega) was added to the pellet, and the mixture was pipetted gently until the cells were resuspended. The mixture was then incubated at 80°C for 5 min to lyse the cells, and then cooled at room temperature. 3 µl of RNase solution (Promega) was added to the cell lysate and, after thorough mixing, the sample was incubated at 37°C for 45 min, and then cooled to room temperature. 200 µl of Protein Precipitation Solution (Promega) was added to the RNase-treated cell lysate, then the mixture was vortexed vigorously at high speed for 20 sec to mix the protein precipitation solution with the cell lysate. Next, the sample was incubated on ice for 5 min, and again centrifuged at 13000 rpm for 3 min. The supernate containing the DNA was carefully transferred to an eppendorf tube (1.5 ml) containing 600 µl of isopropanol at room temperature and mixed gently by inversion until the thread-like strands of DNA formed a visible mass. The sample was centrifuged at 13000 rpm for 2 min, and the supernate was poured off and the tube drained on clean absorbent paper. To the pellet was added 600 µl of 70% v/v ethanol at room temperature and the mixture gently inverted several times to wash the DNA pellet. Then, the sample was centrifuged for 2 min at 13000 rpm. The supernate was carefully aspirated, and the pellet allowed to air-dry for 10-15 min.

The DNA pellet was rehydrated by adding 100µl of DNA Rehydration Solution (Promega) for 1 h at 65°C and periodically mixed by gently tapping the tube. Alternatively, the DNA was rehydrated by incubating the solution overnight at room temperature or at 4°C. Finally, the DNA was aliquoted and stored at 2-8°C or -20°C for longer storage.

2.2.2 Isolation of plasmid DNA

Plasmids used in this study are listed in Table 2.4. The QIA prep[®] Mini prep Purification System (Qiagen) was used for plasmid DNA isolation according to the manufacturer's protocol. A 5 ml volume of an overnight *E. coli* culture in LB (Luria-Bertani) broth was centrifuged at 10000 ×g for 10 min. The bacterial pellet was resuspended in 250 µl of buffer P₁ and transferred to a micro centrifuge tube. No cell clumps should be visible after re-suspension of the pellet. Then, 250 µl of buffer P₂ (alkali-detergent solution) was added, and the tube gently inverted 4-6 times at room temperature to mix. It should not be vortexed, as this will result in shearing of genomic DNA. The tube was inverted until the solution became viscous and slightly clear, but the lysis reaction was not allowed to proceed for more than 5 min. The macromolecules were then precipitated using 350 µl of buffer N₃ (Chaotropic solution) and centrifuged at 13000 rpm for 10 min in a tabletop micro centrifuge. To avoid localised precipitation, the solution was mixed gently, but thoroughly, immediately after addition of buffer N₃, and the solution became cloudy. After centrifugation, a compact white pellet was formed. The supernate from the last step was added to the QIA prep Spin Column by decanting or pipetting, and centrifuged for 30-60 seconds, then the flow-through was discarded. The QIA prep Spin Column was washed by adding 0.5 ml of buffer PB (trace nuclease removal) and centrifuged for 30-60 seconds, and the flow-through was discarded. This wash step was performed only for *endA*⁺ strains, including the JM series such as *E. coli* JM109 as used in this study, or any wild-type strains, which had high levels of nuclease activity or high carbohydrate content. This additional wash step was not used for host strains such as XL-1 blue and DH5α. Then, the QIA prep Spin Column was washed by adding 0.75 ml of buffer PE containing ethanol and centrifuged for 30-60 seconds. The flow-through was discarded and a final centrifuge step was performed for an additional 1 min to remove residual wash buffer. The residual wash buffer was completely removed since the residual ethanol from buffer PE might inhibit subsequent enzymatic reactions. To elute the DNA, 50 µl of buffer EB (10 mM Tris-HCl, pH 8.5) was added to the centre of the QIA prep Spin Column, and let stand for 1 min, then centrifuged for 1 min.

Table 2.4 Plasmids used in this study

Plasmid name	Description	Source
pQE60 (ATG1)	Vector (Qiagen) for expression of recombinant protein with His-tag, containing most of full length of <i>bapC</i> gene	Blackburn PhD thesis, 2000
pSS1129	Suicide vector for gene replacement in <i>B. pertussis</i>	Stibitz <i>et al.</i> , 1986
pbap5-Kana [P-B-5]	Suicide vector pSS1129 containing most of <i>bapC</i> gene, disrupted by insertion of kanamycin resistance cassette	Bokhari PhD thesis, 2002
pCR-Script (Bap-5;Km ^r) [pCR-Script <i>bapC</i> ::Km]	Cloning vector pCR-Script containing most of <i>bapC</i> gene disrupted with kanamycin resistance cassette	Blackburn PhD thesis, 2000
pQE60 (NTS)	Recombinant protein expression vector pQE60 containing gene for part of BapC N-terminus	Blackburn PhD thesis, 2000
pGEMT-Bap5	cloning vector pGEMT containing most of <i>bapC</i> gene	Blackburn PhD thesis, 2000
pSS1129 (<i>bapC</i> ::Tet) [pBapC-Tet(<i>bapC</i> ::Tet)]	Suicide vector pSS1129 containing most of <i>bapC</i> gene, disrupted with tetracycline resistance cassette	This study
pET33b [pET-33b(+)]	Expression vector based on the T7 promoter-driven system (Appendix II)	Novagen
pET33b (BapC + SS)	pET33b expression vector containing full length of <i>bapC</i> gene with predicted signal sequence	This study
pET33b (BapC – SS)	pET33b expression vector containing full length of <i>bapC</i> gene without predicted signal sequence	This study
pBR322	Cloning vector (Appendix II); source of tetracycline resistance cassette	Division of Infection & Immunity, University of Glasgow
pGEMT-Tet	cloning vector pGEMT containing tetracycline resistance cassette	This study

2.3 Agarose gel electrophoresis

2.3.1 Sample preparation

The sample DNA (5-30 µl) was mixed with 6×DNA loading buffer in a 5:1 (vol:vol) ratio prior to loading into the wells. Molecular weight markers (1 kb ladder, Promega) were used.

2.3.2 Gel preparation and electrophoresis

Pre-weighed agarose (TypeII-A medium EEO, Sigma), was suspended in $0.5 \times$ Tris-Borate-EDTA (TBE) buffer (Appendix AI.2.1) and heated until the agarose solution became clear (completely dissolved). The solution was allowed to cool to the extent that it was still warm and ethidium bromide (Bio-Rad, UK) was added to a final concentration of 0.5 µg/ml. A gel tray was prepared by taping the edges with adhesive tape and the gel was cast to the desired thickness. Upon setting, the gel was immersed in 0.5×TBE buffer containing ethidium bromide (0.5 µg/ml) in a horizontal submarine electrophoresis tank. A power pack was used to provide a constant voltage corresponding to 1-5* volts/cm (measured as the distance between the electrodes). Electrophoresis was carried out until the marker dye in the loading buffer had migrated an appropriate distance through the gel.

*During electrophoresis the ethidium bromide migrates toward the cathode (in the direction opposite to that of the DNA). Extended electrophoresis can remove much of the ethidium bromide from the gel, making detection of small fragments difficult. Since sometimes this occurred, the gel was re-stained by soaking it for 30-45 minutes in a solution of ethidium bromide (0.5 µg/ml).

2.3.3 Visualisation of DNA

The ethidium bromide-stained DNA was visualised using a UV transilluminator (model TM-40, UVP Inc., California, USA). Images were stored electronically as appropriate using the Gel Documentation System-Image Store 5000, version 7.2 (UltraViolet Products, Cambridge, UK) as well as printed using a video graphic printer. Electronic images were edited using Adobe PhotoShop 3.0 and images labelled with Microsoft PowerPoint 4.0

2.3.4 Gel extraction procedure

A QIAquick Gel Extraction kit (Qiagen) was used according to the manufacturer's instructions. The DNA fragment of interest was excised from the agarose gel with a clean, sharp scalpel. The size of the gel slice was minimised by removing extra agarose around a band. The gel slice was weighed in a colourless tube and 3 volumes of buffer QG were added to 1 volume of gel (100 mg \approx 100 μ l). For >2% agarose gels, 6 volumes of buffer QG were added. The tubes were incubated at 50°C for 10 min or until the gel slice was completely dissolved. The tubes were mixed by vortexing every 2-3 min during the incubation to help the gel to dissolve. The colour of the mixture should be yellow (similar to the buffer QG without dissolved agarose) after the gel slice was completely dissolved. If the colour of the mixture was violet, 10 μ l of 3M-sodium acetate, pH 5.0 was added and then the colour turned to yellow. The yellow colour indicates the pH \leq 7.5 where the absorption of DNA to the QIAquick membrane in a later step is most efficient.

One gel volume of isopropanol was added to the sample and mixed. This step increases the yield of DNA fragments <500 bp and >4 kb. For DNA fragments between 500 bp and 4 kb, addition of isopropanol had no effect on yield. The maximum volumes of 800 μ l samples were applied to the QIAquick column to bind DNA and then centrifuged at 13000 rpm using a tabletop micro centrifuge (Biofuge, Heraeus, Germany) for 1 min. Sample volumes of more than 800 μ l were simply loaded and spun again. The flow-through was discarded and the QIAquick column placed back in the same collection tube. To remove all traces of agarose, 0.5 ml of buffer QG was added to QIAquick column and centrifuged at 13000 rpm for 1 min. To wash the column, 0.75 ml of buffer PE was added to QIAquick column and left for 2-5 min and then centrifuged at 13000 rpm for 1 min. 2-5 min incubation time was used for salt-sensitive applications, such as blunt-ended ligation and direct sequencing.

The flow-through was discarded and, to remove completely the residual ethanol from buffer PE, the QIAquick column was centrifuged for an additional 1 min at 13000 rpm. The QIAquick column was placed into a clean 1.5 ml centrifuge tube and 50 μ l buffer EB added to the centre of the QIAquick membrane and the column was then centrifuged at 13000 rpm for 1 min to elute DNA. The average eluate volume was 48 μ l from 50 μ l elution buffer volume, and the maximum elution efficiency was achieved between pH 7.0 and 8.5.

2.4 Restriction enzyme reactions

Restriction enzymes or endonucleases cleave double-stranded DNA (dsDNA) at specific sites within or adjacent to their recognition sequences. All reaction were performed based on the manufacturer's instructions (Promega). Total reaction was performed in a volume of 20 μ l on 0.2-1.5 μ g of substrate DNA, using usually 5-fold excess of enzyme over DNA. In a sterile tube, reagents were added in order as follows: sterile deionized water 16.3 μ l, restriction enzyme 10 \times buffer 2 μ l, acetylated BSA (to enhance the enzyme activity) 0.2 μ l of 10 μ g/ μ l, DNA 1 μ l of 1 μ g/ μ l, and mixed by pipetting, then the restriction enzyme 0.5 μ l of 10u/ μ l was added. It was then mixed gently by pipetting and centrifuged for a few second and finally incubated at the optimum temperature for 1-4h. 4 μ l of 6 \times loading buffer was added prior to gel analysis. Where appropriate, the enzymes were heat-inactivated at 65°C or 80°C for 15 min according to manufacturer's instructions.

2.5 Cloning protocol

2.5.1 DNA preparation

Vector and insert DNA were subjected to restriction endonuclease digestion to obtain complimentary cohesive overhangs and the resultant fragments were gel purified. DNA concentrations were estimated and ratios determined according to the equation (1) and (2), respectively.

Equation 1:

$$\text{Concentration of DNA (ng/\mu l)} = A_{260\text{nm}} \times 50 \times \text{dilution factor}$$

For quantitating the amount of DNA, readings were taken at wavelengths of 260 nm and 280 nm. The reading at 260 nm allows calculation of nucleic acid in the sample. An OD of 1 corresponds to approximately 50 μ g/ml for double-stranded DNA, 40 μ g/ml for single-stranded DNA, and ~20 μ g/ml for single-stranded oligonucleotides. The ratio between the readings at 260 nm and 280 nm ($\text{OD}_{260}/\text{OD}_{280}$) provides an estimate of the purity of the nucleic acid. Pure preparations of DNA have $\text{OD}_{260}/\text{OD}_{280}$ values of 1.8. If there is contamination with protein or phenol, the $\text{OD}_{260}/\text{OD}_{280}$ will be significantly less than the values given above, and accurate quantitation of the amount of nucleic acid will not be

possible. Alternatively, band intensity of a sample of DNA was compared with those of molecular weight standards of known concentration after agarose gel electrophoresis.

Equation 2:

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{Molar ratio of} \frac{\text{Insert}}{\text{Vector}} = \text{ng of insert}$$

Insert: Vector ratios of 1:1 and 3:1 were commonly utilised.

2.5.2 Ligation strategies

Ligation reactions were performed in a total volume of 10 µl using 3 units of T4 DNA ligase and ligase 10×buffer (Promega, USA). After incubation at 16°C for 18h for a blunt-end ligation, or at room temperature (22°C) for 3h or 4-8°C overnight for a sticky-end ligation, the ligation was terminated by heating the reaction mix at 70°C for 10 min, and then the products were stored at –20°C until use. The correct and complete ligation was analysed by gel electrophoresis using unligated material as a suitable control.

2.6 Transformation

2.6.1 Preparation of electroporation-competent cells

A 2-L dimpled flask containing 500 ml of fresh LB broth was inoculated with 5 ml of an overnight culture of *E. coli* strain. The flask was incubated at 37°C on a shaker until an OD_{600nm} of 0.5-0.7 was obtained. The flask was then chilled on ice for 30 min and thereafter harvested at 2500 ×g (10000 rpm) for 15 min in a Sorvall superspeed (rotor GS-3). The resultant bacterial pellet was resuspended in 500 ml of cold sterile distilled water and then centrifuged for 15 min as mentioned previously. The cell pellet was resuspended in 250 ml of cold sterile distilled water and then centrifuged for 15 min as previously described. The cell pellet was suspended in 10 ml of cold sterile glycerol 10% (v/v) in distilled water and then the final centrifugation performed for 15 min, as before. The cell pellet was resuspended in 1.5 ml of sterile 10% (v/v) glycerol and 40-100 µl aliquots were snap frozen in liquid nitrogen or on dry ice. Finally, the aliquots were stored at -70°C. Cells are usable

for approximately 6 months. For use, cells were kept cold (on ice) at all times during the procedure to be as close to 0°C as possible.

2.6.2 Electroporation procedure

Electroporation cuvettes (0.1 cm or 0.2 cm) (Bio-RAD, UK) and the safety chamber were previously chilled at -20°C. To chilled eppendorf tubes, 1-5 µl of DNA (ligation mix or plasmid preparation) and 40 µl of thawed competent cells on ice were added. The mixture was gently swirled (to avoid multiple pipetting) and incubated on ice for 10-20 min, then transferred to pre-chilled cuvettes on ice. The cuvettes were gently tapped to deposit the liquid at the bottom of the tube, and placed in a Bio-Rad Gene Pulser (model 1652098, Bio-Rad laboratories, UK) set at 1.5 KV, 25 µFD, and 200 Ω for 0.1 cm or 2.5 KV, 25 µFD, and 200Ω for 0.2 cm. The cells were pulsed once for 4-5 msec. At no time were the cells exposed to room temperature except for a few seconds. Immediately following electroporation, 1 ml of pre-warmed (37°C) SOC medium (Appendix AI.1) was added to the cells and incubated for 1-2 h at 37°C with shaking at 225-250 rpm. This growth step before spreading the transformation mixture onto plates is usually necessary for efficient transformation when non-lactamase selection markers are used. Finally, various volumes of the mixture (e.g. 10 µl, 100 µl) were plated on selective LB agar containing the appropriate antibiotics, and incubated overnight at 37°C.

2.6.3 Preparation of competent cells for heat shock transformation

A 1ml overnight culture was inoculated into LB broth and grown for about 3h at 37°C in a shaking incubator until the OD₆₀₀ was approximately 0.5-0.7. The culture was then cooled on ice and cells pelleted by centrifugation at 2500 ×g for 10 min at 4°C in a Sorvall RT 6000B refrigerated centrifuge. The cells were resuspended in 50 ml of cold 50 mM CaCl₂ in distilled water and left on ice for 30 min. Cells were then pelleted as before and resuspended in 10 ml of cold 50mM CaCl₂ , 15% (v/v) glycerol. 200 µl aliquots were snap frozen and stored at -70°C.

2.6.4 Heat shock transformation

Competent cells from -80°C prepared for heat shock transformation were thawed on ice for 10 min. 5 µl of each ligation reaction was added to 100 µl competent cells and incubated on

ice for a further 30 min. The cells were then heat shocked by transferring to a water bath at 42°C for 45 sec, then returned to ice for 2 min before addition of 950 µl of SOC medium. The cultures were incubated at 37°C with shaking for 1 h and then 100 µl of each was plated on LB agar containing appropriate antibiotics. Plates were incubated at 37°C overnight and next day were checked for the appearance of colonies.

2.7 Bacterial conjugation and gene replacement

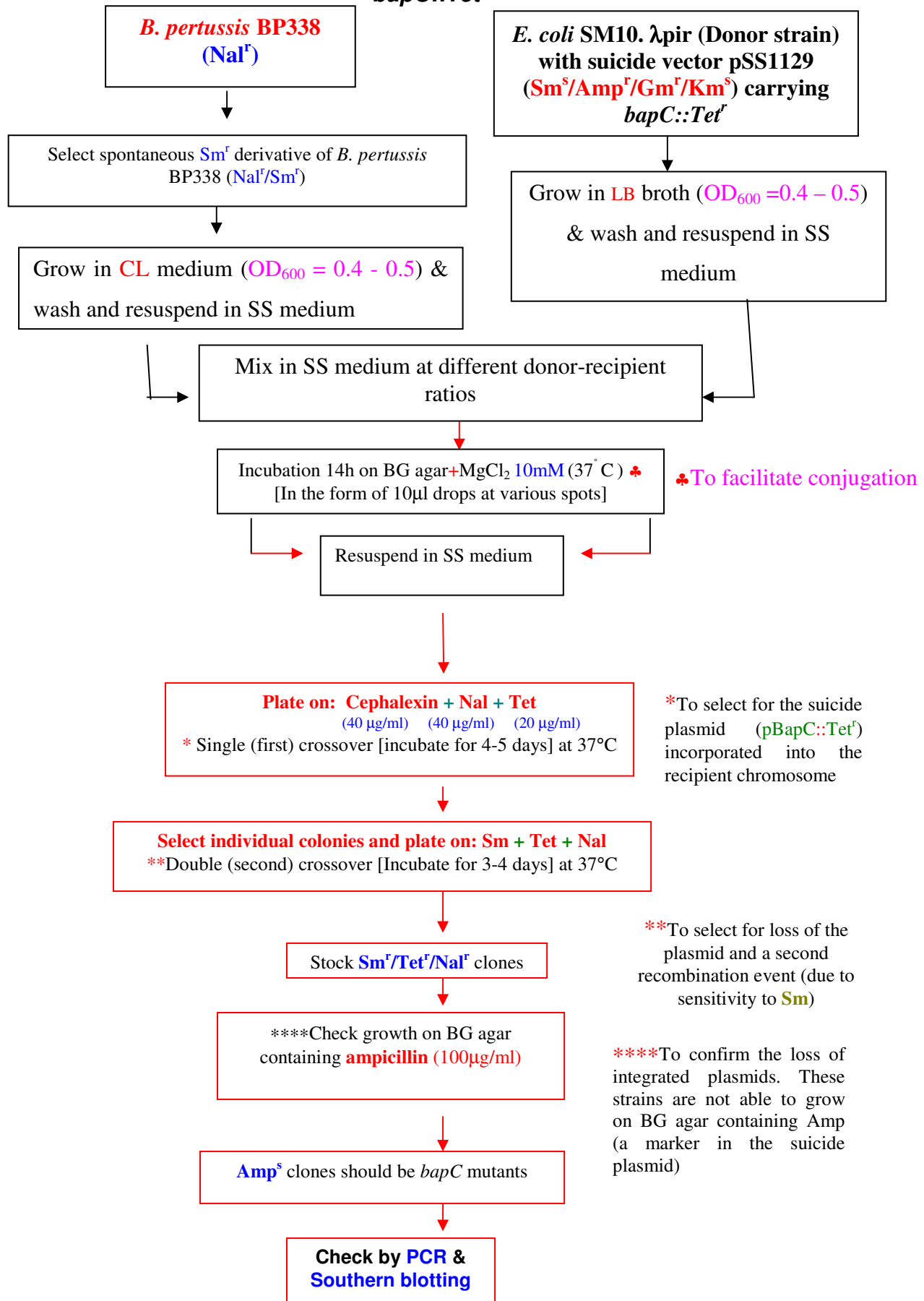
2.7.1 Construction of *BapC* mutant of BP338

The replacement of a chromosomal gene with its *in vitro*-altered counterpart by homologous recombination is a standard method for use with *B. pertussis*. The most widely-used suicide vector, pSS1129, is a derivative of pRTP1, and was constructed by Stibitz *et al.* (1986) (Table 2.4).

Bacterial conjugation was carried out by plate mating on BG agar plates between *E. coli* SM10.λpir (donor strain)(a DNA mobilising strain) carrying, on pSS1129, the mutated *bapC* gene, disrupted with the tetracycline cassette (Table 2.4), and *B. pertussis* BP338S (Table 2.2) (see Fig. 2.1).

B. pertussis BP338S and *E. coli* SM10.λpir with pSS1129 (*bapC::Tet*)(donor strain) were grown on BG agar containing 15% v/v horse blood and streptomycin (100 µg/ml), and LB agar containing tetracycline (20 µg/ml), respectively. Colonies of *B. pertussis* were inoculated into 10 ml of CL medium (Appendix I.1) and grown at 37°C for 14-18 h. Colonies of *E. coli* were inoculated into 10 ml of LB broth and incubated at 37°C for 3-4 h. The resulting OD_{600nm} for *E. coli* SM10.λpir in two different experiments was 0.4 (1×10⁸ CFU/ml) and 1.0, and for *B. pertussis* was 0.5 (~early logarithmic phase) and 0.8, respectively. The concentration of *B. pertussis* was adjusted by comparison with an opacity rod (5th International Reference of Opacity, Perkins *et al.* 1973) to 10 opacity units i.e.~ 10⁹ CFU/ml. 1 ml volumes of *E. coli* and *B. pertussis* cultures were then separately centrifuged in a tabletop micro centrifuge (Biofuge, Heraeus, Germany) at 13000 rpm for 1 min.

Figure 2.1 Construction of *bapC* mutant of *B. pertussis* by conjugation of BP338 strain with *E. coli* carrying suicide vector pSS1129 containing *bapC::Tet^r*



Materials & Methods

The supernates were aspirated and pellets resuspended and washed twice in 1 ml of respective media (at the same speed as above for 5 min). The pellets of both donor and recipient were resuspended in 100 µl of CL medium and mixed together in a 1.5 ml eppendorf tube at different donor: recipient ratios such as 1:1, 1:2, 1:3, 1:4, 1:10 and 1:100. Aliquots of 10 µl of mixture were plated on freshly-made and dried BG agar containing 10 mM MgCl₂, which facilitates conjugation, in the form of drops, and were incubated at 37°C for 3-14 h (Stibitz, 1994).

The bacterial cells were then collected from the agar surface with a loop and resuspended in SS-X medium, and then washed and resuspended in 100 µl fresh SS-X medium. To select for the first (single) crossover, where the suicide plasmid is incorporated into the *B. pertussis* chromosome, bacterial suspensions were then plated onto BG agar plates containing cephalixin (40 µg/ml), nalidixic acid (40 µg/ml) and different tetracycline concentrations (2, 3, 5 µg/ml). The plates were incubated at 37°C for 5-6 days to select the exconjugants. The cephalixin was included to select against growth of the *E. coli* donor as *B. pertussis* is naturally resistant to cephalixin (40 µg/ml).

The second crossover was obtained by selecting for the loss of the integrated plasmid. The exconjugants were plated on BG agar plates supplemented with streptomycin (100 µg/ml), nalidixic acid (40 µg/ml), and tetracycline (2, 3, 5 µg/ml), and were incubated at 37°C for 5-6 days: The suicide vector pSS1129 has the *rspL* gene which encodes streptomycin sensitivity and only those bacteria that have lost the plasmid will be able to grow.

To confirm the loss of integrated plasmid, the survivors of the above selection were grown on BG agar containing ampicillin (100 µg/ml), a marker on the suicide plasmid (pSS1129). Survivors of streptomycin selection that are ampicillin sensitive show that they have lost the vector (Fig. 2.1). Colonies of *B. pertussis* that are Tet^R, Sm^S should have the *bapC* allele replaced with the *bapC::Tet^R* allele.

2.7.2 Construction of *BrkA*⁻, *BapC*⁻ double mutant

Construction of *BrkA*⁻, *BapC*⁻ double mutant was performed under the same conditions for making BP338 (*BapC*⁻) except in some steps. Bacterial conjugation was carried out by plate mating the *E. coli* SM10.λpir (donor strain) carrying on pSS1129 the mutated *bapC* gene disrupted with the tetracycline cassette and *B. pertussis* BP2041S (*brkA* mutant). *B. pertussis* BP2041S was grown on BG agar containing 15% v/v horse blood and kanamycin

(40 µg/ml). For the first (single) crossover, where the suicide plasmid is incorporated into the *B. pertussis* chromosome, bacterial suspensions were plated onto BG agar plates containing kanamycin (40µg/ml), cephalexin (40 µg/ml), nalidixic acid (40 µg/ml) and different tetracycline concentrations (2, 3, 5 µg/ml).

The second crossover was performed by selecting for the loss of the integrated plasmid via plating the exconjugants on BG agar plates supplemented with streptomycin (100 µg/ml), kanamycin (40 µg/ml), nalidixic acid (40 µg/ml), and tetracycline (2, 3, 5 µg/ml), and incubation at 37°C was continued for 5-6 days:

2.8 Polymerase chain reaction

The PCR conditions were optimised, where necessary, according to the orthogonal array method described by Cobb *et al.* (1994). The annealing and elongation thermal parameters were then adjusted to obtain optimal conditions. A Hybaid thermal cycler was used for all reactions. The products of the PCR were stored at –20°C or used immediately.





2.8.1 Primers

During primer design, care was taken to avoid potential internal secondary structure, and where possible, a GC clamp was engineered at the 3`end of the primer. Additionally, primers were checked to avoid overlapping regions and possible dimerisation, potential for secondary structure formation and for compatible T_m values. T_m values were calculated according to the equation:

$$T_m^{\circ}\text{C} = 4 (G+C) + 2 (A+T) - 5^{\circ}\text{C}$$

Primers were obtained from Invitrogen and resuspended in filter sterilized distilled water to give a final concentration of 100 pmol/µl or 25 pmol/µl for PCR (Details of primers used in this study are given in Table 2.5).

Table 2.5 Primers used in this study

Primer designation	Primer sequence	Comments
<i>bapC</i> forward 5' to 3' (BAP5F)	ATGGCACCTCGCCTTCGATTGCGTCCAAG	Amplifies <i>bapC</i> gene from nucleotide 728 (see Appendix II)
<i>bapC</i> reverse 3' to 5' (BAP5R)	AGGTGGAACGTCCAAGGCAAGGTCAGCTTG	Amplifies <i>bapC</i> gene from nucleotide 2960
Forward (5' to 3') primer of tetracycline-resistance gene (TCF1)	AATC/CATGGTTTCTCATGTTTGACAGCTTATCATCG 	Amplifies <i>Tet</i> cassette from pBR322 with a designed <i>NcoI</i> recognition sequence
Reverse (3' to 5') primer of tetracycline-resistance gene (TCR1)	ACGC/CATGGTTTGCGCATTACAGTTCTCCGC 	Amplifies <i>Tet</i> cassette from pBR322 with a designed <i>NcoI</i> recognition sequence
Forward (5' to 3') primer of <i>bapC</i> gene with signal sequence (BAPCF14)	TAATAATCATATGAATGACAGAAAATCCAATAGC 	Amplifies full length <i>bapC</i> gene from first codon of the signal sequence and with a designed <i>NdeI</i> recognition sequence
Forward (5' to 3') primer of <i>bapC</i> gene without signal sequence (BAPCF24)	TAATAATCATATGCAGGCCGCGCCGGCCGCCGCC 	Amplifies <i>bapC</i> gene after signal sequence with a designed <i>NdeI</i> recognition sequence

Materials & Methods

Reverse (3' to 5') primer of <i>bapC</i> gene (BAPCR12)	ACGGATCCGACTACCAGGTGTAGCGATAGCCC  <i>Bam</i> HI recognition sequence	Amplifies <i>bapC</i> gene from nucleotide 2982 with a designed <i>Bam</i> HI recognition sequence
Forward (5' to 3') primer of <i>bapC</i> gene without signal sequence (BAPCF25)	AATAATGCTAGCATGCAGGCCGCGCCGGCCGCGCCG  <i>Nhe</i> I recognition sequence	Amplifies <i>bapC</i> gene from first codon after signal sequence and with a designed <i>Nhe</i> I recognition sequence
Forward (5' to 3') primer of <i>bapC</i> gene with signal sequence (BAPCF15)	AATAATGCTAGCATGAATGACAGAAAATCCAATAGC  <i>Nhe</i> I recognition sequence	Amplifies full length <i>bapC</i> gene from first nucleotide of the signal sequence and a designed <i>Nhe</i> I recognition sequence
Forward (5' to 3') primer of <i>bapC</i> gene (MNF1)	ATGAATGACAGAAAATCCAATAGC	Amplifies <i>bapC</i> gene from first codon of the signal sequence to determine the region encompassing poly(C) tract
Reverse (3' to 5') primer of <i>bapC</i> gene (MNR1)	GCTGTCACGCACGGTGAGCGAACG	Amplifies <i>bapC</i> gene from nucleotide 408
Forward (5' to 3') primer of <i>bapC</i> gene (MNF2)	CGTGTATGTCGACTCGGAGCACCG	Amplifies <i>bapC</i> gene from nucleotide 363 to determine the region encompassing poly(G) tract
Reverse (3' to 5') primer of <i>bapC</i> gene (MNR2)	CCAGACCCTGTTGCCACCACAGCG	Amplifies <i>bapC</i> gene from nucleotide 818
Forward (5' to 3') primer of <i>brkA</i> gene (BRKF)	CGGTTGACCGACGGCGCCACGGCGC	Amplifies <i>brkA</i> gene from nucleotide 2701 (see Appendix II.4)

Materials & Methods

Reverse (3' to 5') primer, in Tn5 (TN5R1)	ATGCGCCCACTGCGCAGGCTCAAGC	Amplifies <i>Tn5</i> gene from nucleotide 4919
Reverse (3' to 5') primer, in Tn5 (TN5R2)	CACCACGAAGCGCTCGTTATGCGCC	Amplifies <i>Tn5</i> gene from nucleotide 5101
Reverse (3' to 5') primer, in Tn5 (TN5R3)	GTGGTGCTTCGCGAGCAATACGCG	Amplifies <i>Tn5</i> gene from nucleotide 718
Forward (5' to 3') primer of <i>brkA</i> gene (BRKAF2)	AACTGCCGGGTGAAGACGGCGCCG	Amplifies <i>brkA</i> gene from nucleotide 61
Reverse (3' to 5') primer of <i>brkA</i> gene (BRKR2)	AAGCTGTAGCGGTAGCCGGCGTGG	Amplifies <i>brkA</i> gene from nucleotide 4238

2.8.2 HotStar Taq™ PCR Method

Hot-start PCR was performed according to the HotStar Taq™ PCR kit manufacturer's instructions (Invitrogen). The following mixture was prepared in a thin-walled 0.5 ml tube immediately before use.

Mixture per reaction (μl)

10×PCR buffer (containing 15 mM MgCl ₂)*	5	
5× Q solution**	10	
10 mM dNTPs	1	
Primer 1 (Forward) [25 pmol/μl]	2	
Primer 2 (Reverse) [25 pmol/μl]	2	
Hot Star Taq DNA polymerase [5 units/μl]	0.2	
dH ₂ O (filter sterilized)[for negative control = 29.8 μl]	28.8	
Template DNA	1	
<hr/>		
Total volume in each PCR tube		50

* Final concentration of MgCl₂ is 1.5 mM.

** Q solution facilitates amplification of difficult templates by modifying the melting behaviour of DNA. This reagent will often enable or improve a suboptimal PCR caused by templates that have high degree of secondary structure or that are GC-rich.

The following thermo-cycling parameters were used:

	<u>Time</u>	<u>Temperature</u>
Initial activation step:	10 min	95°C
30 cycles of:		

Denaturation:	1 min	94°C
Annealing:	1 min	56°C
Extension:	4 min	72°C
Final extension time:	10 min	72°C

2.8.3 Touchdown PCR

Touchdown PCR is a simple method to optimize yields of amplified DNA when the melting temperature of hybrids between the oligonucleotide primers and their target sequences is not known with certainty, and spurious smaller bands appear in the product spectrum.

The earliest steps of a touchdown PCR cycle have high annealing temperatures (least-permissive of non-specific binding) and, every subsequent cycle, the annealing temperature is decreased by 1 or 2 degrees Celsius. In this study, after initial denaturation at 95°C for 5 min, the reaction mix in the earliest steps of a touchdown PCR cycle had a high annealing temperature of 64°C for 1 min. For every subsequent 5 cycles, the annealing temperature was decreased by 2°C. The final 10 cycles were then performed at 54°C to make a total of 35 cycles. The cycling conditions for denaturation at 95°C for 1 min, annealing for 1 min and elongation at 72°C for 3 min were used before a final elongation step at 72°C for 10 min.

2.8.4 Colony PCR

Colony PCR is the screening of bacterial (*E. coli*) clones for correct ligation or plasmid products. Selected colonies of bacteria were picked with a sterile toothpick or pipette tip from a growth plate (LB agar with appropriate antibiotic), and swirled into 25 µl of TE (Appendix AI.2.4) in an autoclaved microfuge tube. The area of picked bacteria was marked and (preferably) numbered on the bottom of the plate (and the microfuge tube). The mix tube was heated in a heating block at 90-100°C for 10 min, and then the samples were incubated at -20°C for 5 min and centrifuged for 10 min at 13000 rpm. 20 µl of the supernatant was then transferred into a new microfuge tube and 1-2 µl of the supernatant

was used as template in a 50 µl PCR standard reaction. PCR was conducted to determine if the colony contained the DNA fragment or plasmid of interest.

2.8.5 Nested PCR

Nested PCR means that two pairs of PCR primers were used for a single locus. The first pair amplified the locus as in any PCR experiment. The second pair of primers (nested primers) bind within the first PCR product, and produce a second PCR product that will be shorter than the first one. The logic behind this strategy is that if the wrong locus was amplified by mistake, the probability is very low that a second pair of primers would also amplify it a second time. The thermo-cycling parameters for both external and internal PCR rounds were 1 cycle of 94°C for 5 min; 30 cycles of 94°C/1 min, 60°C/1 min and 72°C/3 min and a final extension time at 72°C for 7 min.

2.8.6 Multiplex PCR

In order to develop a multiplex PCR system for the simultaneous detection of different gene fragments in the target, the following mixture was prepared. Each of the 0.5 ml eppendorf tubes contained 2 µl of 10 mmol l⁻¹ dATP, dTTP, dGTP and dCTP, 5 µl of 10×PCR buffer containing 15 mmol l⁻¹ MgCl₂, 10 µl of 5×Q solution, 25 pmol of each primer and 2 µl of target DNA. After adding 0.5 µl of DNA Taq polymerase, the final volume of the mixture was then adjusted to 50 µl by adding sterilised distilled water. The following thermo-cycling parameters were used; 94°C for 4 min followed by 35 cycles of PCR (94°C, 1 min; 60°C, 30s; and 72°C, 1 min for denaturation, annealing and extension in each cycle). Final extension was performed at 72°C for 10 min.

2.8.7 Purification of PCR products

A QIAquick PCR purification kit was used according to the manufacturer's instructions (Qiagen). This protocol is used to purify fragments ranging from 100 bp to 10 kb away from primers, nucleotides, polymerases, and salts.

5 volumes of buffer PB were added to 1 volume of the PCR sample and mixed. The sample was then applied to the QIAquick column to bind DNA, and this was centrifuged by tabletop micro centrifuge (Biofuge, Heraeus, Germany) for 1 min at 13000 rpm. The flow-through was discarded and the QIAquick column returned to the same tube. To the

QIAquick column was added 0.75 ml buffer PE to wash the column and this was centrifuged for 1 min at 13000 rpm. The flow-through was discarded and the column was again centrifuged for an additional 1 min to completely remove residual ethanol from the column. The column was placed in a clean 1.5 ml microcentrifuge tube and 30-50 µl buffer EB was added to the centre of membrane to elute DNA. The column was left for 1 min and then centrifuged by table top micro centrifuge (Biofuge, Heraeus, Germany) for 1 min at 13000 rpm.

2.9 DNA sequencing

Plasmid from *E. coli* M15pQE60 and pET-33b (+) expression vectors carrying a part or the whole of *bapC* gene, respectively, were purified using the QIA prep[®] Mini prep purification kit. Appropriate concentrations of primers (*bapC* forward and *bapC* reverse) (Table 2.5) and plasmids were sent to the Molecular Biology Support Unit (MBSU) at the University of Glasgow to perform DNA sequencing. A MegaBACE1000 (96 capillary) sequencer, which used Big Dye (Applied Biosystems) and ET-Dye Terminator (Amersham Bioscience) chemistries, was employed. The reaction chemistry was based on the dideoxy method developed by Sanger *et al.* (1977). Each dideoxy nucleotide contained a specific fluorescent dye that could be excited by a laser. The signals were then collected and analysed by specific software. A coloured electropherogram was produced. The data were downloaded and saved on to a CD. For data analysis, Chromas (versions 2.3 & 1.15) and DNASTAR softwares were utilised. Preparations of clean template DNA produced a read of around 600 bases, including 400-600 bases of accurate sequence. The length of accurate sequence is primarily determined by the quality of the template DNA and the efficiency of primer annealing.

2.10 Southern blotting

The term ‘Southern blotting’ is commonly used to encompass DNA transfer from any type of gel to any type of filter matrix. The DNA samples were separated by electrophoresis through an agarose gel and stained with ethidium bromide and then the gel photographed. The DNA was depurinated and denatured within the gel prior to transfer. The depurination was accomplished by treating the gel in 0.25M hydrochloric acid for 10 min in an orbital shaker at room temperature, and then rinsing the gel thoroughly twice in dH₂O.

Depurination of the DNA in acid makes the DNA more flexible and is critical for complete transfer of fragments of > 4kb which would otherwise diffuse poorly through the gel. However, it has been noted that the depurination is apparently unnecessary for the transfer of small (<10-kb) fragments (Boehringer Mannheim). Moreover, excessive depurination is very damaging to the DNA in the gel. The gel was allowed to float in denaturing solution for 2×15 min with gentle shaking followed by rinsing it twice with dH₂O. It was then submerged for 2×15 min in neutralising solution 20×SSC (Appendix A1.4).

For transfer, a typical blotting apparatus was set up using a clean reservoir filled with at least 500 ml of 20×SSC. A flat platform was set up and covered with three wet sheets of Whatman 3MM in 20×SSC. The ends of the 3MM paper dipped into the 20×SSC reservoir and served as a wick. Air bubbles were removed by rolling a clean pipette over it. The neutralised gel was placed on the saturated 3MM paper on the level platform and any air bubbles that might be trapped between the paper and the gel were removed.

The exact size of nylon membrane (Hybond-N, Amersham) was cut and soaked by laying it on the surface of distilled water and then submerging. The water was poured off and the filter soaked in 20×SSC, and then placed on the top of the gel. Any air bubbles were removed and Saran wrap or parafilm was placed around the edges of the gel so that no part of the membrane could directly contact the 3MM paper wick. Five sheets of dry 3MM paper were placed on the top of the filter and stacked paper towels to a thickness of 4cm on top of the 3MM paper. A glass plate was put on top of the paper towels and a small weight (approx. 500-750g) added to the top. This was left overnight to blot by capillary action.

After disassembling the blotting set up, the position of wells on the filter were marked and one corner was cut to allow orientation of the gel. The nylon membrane was carefully peeled off and gently submerged in a solution of 2×SSC for 3 min to remove any agarose particles and excess salt and then it was wrapped in cling film. The filter should still be a clear white at this stage since patches of yellow on the filter that have appeared during the blot indicate that the gel was incompletely neutralised before transfer. The gel was then re-stained with ethidium bromide to check that most of the DNA had been transferred. DNA was then crosslinked to the wet membrane without prior washing, after exposure to a UV cross linker (Spectrolinker XL-1000, Spectroline, New York, USA) for about 1 min.

After the UV cross-linking, the membrane was briefly rinsed in dH₂O and allowed to air-dry. The membrane was either utilised immediately for prehybridisation or stored at +4°C

for future use. The prehybridisation was performed for 4-6 h in a rolling hybridisation oven (model HB-1D, Techne, Cambridge, UK) with at least 20 ml of prehybridising solution (Appendix AI.4). The temperature of the oven was set at 65°C for prehybridisation. This temperature was maintained for the subsequent hybridisation and washing steps.

2.10.1 Preparation of digoxigenin-labelled probes

The digoxigenin (Dig)-labelled probes were synthesised using a PCR Dig Probe Synthesis Kit (Roche). The PCR Dig-labelling kit is provided with the reagents required for the direct digoxigenin (Dig)-labelling of DNA fragments generated by the polymerase chain reaction (PCR) process.

The kit enables the synthesis of highly sensitive probes by incorporation of DIG-dUTP into a PCR product. However the purity of template DNA is less critical with this method compared to other methods and, in this study, genomic DNA was used and extracted by the genomic DNA purification kit (Promega). The nucleotide mixture contains dATP, dCTP, dGTP, dTTP, DIG-11-dUTP. Due to multiple incorporation of DIG-dUTP during the PCR process, the molecular weight of the PCR product is increased significantly compared to the unlabelled PCR product. A 1:6 ratio of DIG-dUTP:dTTP was used to amplify the fragments larger than 1kb since these fragments were poorly amplified when high concentrations of DIG-dUTP were utilised as the polymerase is slowed by the presence of the DIG hapten.

According to the manufacturer's instructions, the components were mixed in microcentrifuge tubes on ice and run in a thermal cycler with the following parameters: initial denaturation at 95°C for 2 min, 30 cycles of; denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec and elongation at 72°C for 2 min, and then one cycle final elongation step at 72°C for 7 min.

The primer sequences *brkA* Forward and *brkA* Reverse (Table 2.5), which cover a 1500 bp in the middle of the *brkA* gene were utilised. However the PCR-generated probes are very pure and can be used directly in the hybridisation reaction, the PCR product was purified using the PCR product purification kit as previously described (section 2.8.7). The contents of the labelling tube were then stored at -20°C for future use or denaturation of the probe was performed for 10 min in a boiling water bath followed by immediate snap-cooling on ice just before use.

The probe was then diluted in prehybridization solution for optimal probe concentrations (5-25 ng/ml). The standard probe concentration is 2 µl of probe per ml of hybridisation buffer. In the case of a faint labelled PCR product band on the evaluation gel, up to 4 µl of probe per ml of hybridization buffer was used, and if the labelled band was very strong only 1 µl or even as little as 0.5 µl of probe per ml of hybridization buffer was used.

2.10.2 Probe hybridisation

The prehybridization of the membrane was performed at 65°C-68°C for 4-6 h in 1 ml of pre-warmed prehybridization buffer (Appendix AI.4) per 10 cm² of filter. The filter should be able to move freely in the roller tube or sealed bag and the solution should sweep gently over the surface of the filter. The prehybridising solution was then discarded and the denatured Dig-labelled probe in hybridisation buffer immediately added to the roller tube carrying the DNA samples cross-linked to the nylon membrane.

Hybridization was performed overnight (12-14 h) at 65°C-68°C. The probe was decanted into a universal tube and stored at -20°C. The hybridization solution contains unannealed Dig-labelled probe and can be reused in future hybridization experiments. The membrane was washed twice, 5-15 min per wash, in 50 ml of 2×SSC / 0.1% (w/v) SDS solution at room temperature (low stringency washing). It was followed by 2×15 min washes in 50 ml of 0.1×SSC / 0.1% (w/v) SDS at 65°C-68°C [according to the manufacturer's instructions, for long probes (>100 bp) the membrane was washed at 68°C]. These washes (low and high washes) remove unbound probe, which will lead to high backgrounds if not removed. For most applications, washing in 0.5 × washing solution (Appendix AI.4) is stringent enough. It was determined empirically whether it was necessary to wash with 0.1× wash solution.

2.10.3 Colourimetric detection with BCIP/NBT

Detection was performed according to the protocol described in the Dig system User's Guide for Filter Hybridization (Boehringer Mannheim). After hybridization and post-hybridization washes, the membrane was equilibrated in washing buffer (Appendix AI.4) for 1-2 min. The membrane was then incubated in blocking buffer (Appendix AI.4) for 30-60 min. The anti-digoxigenin-AP (Fab fragments from an anti-digoxigenin antibody from sheep, conjugated with alkaline phosphatase) was centrifuged by tabletop micro centrifuge (Biofuge, Heraeus, Germany) for 5 min at 13000 rpm in the original vial prior to each use,

to remove any aggregates and then the necessary amount was pipetted carefully from the surface of the vial.

The anti-digoxigenin-AP was diluted to 1:5000 (after centrifugation) in blocking buffer for a working concentration of 150 mU/ml (2 µl per 10 ml) and mixed gently by inversion. The blocking buffer was poured off, and the membrane incubated for 30 min in at least 20 ml of the prepared antibody solution at room temperature. The antibody solution was discarded and the membrane washed twice, 15 min per wash, in 100 ml of washing buffer. The membrane was then equilibrated in 20 ml of detection buffer (Appendix AI.4) for 2 min. The detection buffer was poured off, and approximately 10 ml ready mixed BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate / nitro blue tetrazolium) (Sigma) (a solution for detection of alkaline phosphatase-coupled immune complexes) added to the membrane in a plastic bag sealed without bubbles, and then incubated in the dark for 15-45 min at room temperature. The container was not shaken while the colour was developing. Once the desired bands were detected, the membrane was washed with distilled water to prevent over-development.

2.10.4 Stripping membrane for reprobing

Stripping of blots where DNA fragments have been detected colourimetrically was only possible when nylon membranes were used for blotting, since using DMF (dimethylformamide) to remove the colour precipitate will dissolve nitrocellulose under such conditions. A large glass beaker of dimethylformamide was heated to 50-60°C in a water bath. The membrane was incubated in the heated DMF until the colour was removed. Changing the DMF solution frequently will increase the speed of decolourization. The membrane was thoroughly rinsed and washed in dH₂O for 1 min, and not allowed to dry prior to probe removal. The membrane was then incubated twice for 10 min in alkaline probe-stripping solution (Appendix I) at 37°C. This incubation removes the alkali-labile Dig-labelled probe. The membrane was thoroughly rinsed in 2×SSC solution, and reprobing with the prehybridization step of the desired hybridization procedure was performed.

2.11 Protein analysis

2.11.1 Fractionation of bacterial cells

Bordetella strains were cultured on BG agar plates containing 15% (v/v) horse blood and incubated for 48-72 h at 37°C. Ten ml volumes of CL medium were inoculated and incubated for 48 h on a shaker (120 rpm) at 37°C. Two litre dimpled flasks containing 1000 ml of CL medium were pre-warmed overnight at 37°C. Ten ml volumes of the 48 h culture were added to the 1000 ml volumes of pre-warmed CL medium and incubated until mid-log phase (approx. 48 h) on an orbital shaker (120 rpm) at 37°C.

Bacterial cells were harvested in 500-ml Nalgene centrifuge bottles by centrifugation at $5000 \times g$ for 20 min at 4°C in a Sorval superspeed centrifuge (rotor GS-3). Cells were resuspended in envelope buffer (10 mM sodium phosphate, pH 7.2) with a total volume not exceeding 50 ml. The resuspended cells were transferred to 50 ml Nalgene tubes and kept on ice overnight. The cells were then washed by centrifuging at $5000 \times g$ in a Sorval superspeed centrifuge (rotor GS-3) for 30 min at 4°C and resuspended in approximately 8 ml of ice-cold 10 mM sodium phosphate (pH 7.2). The final volume must not exceed 10 ml and cells must be kept on ice. The cells were disrupted on ice using a cell sonicator (probe SM 05: 12 microns amplitude) in 10 sec intervals for 5 min. The sonicated samples were poured into clear 10 ml Nalgene tubes which were placed in iced water for 10 min and then on ice until ready. The samples were then centrifuged at $5000 \times g$, in the same centrifuge and rotor as above, for 30 min at 4°C to pellet large debris, unbroken cells and insoluble materials.

The supernate was carefully removed using long-form Pasteur pipettes and transferred to 10-ml ultracentrifuge tubes. The samples were then centrifuged at $100,000 \times g$ at 4°C for 1 h in a Sorvall ultracentrifuge to pellet the cell envelopes. The resulting supernate contained the soluble content of the cytoplasm and periplasm.

The supernate was collected and the gelatinous pellet thoroughly resuspended in approximately 7 ml of envelope buffer containing sodium *N*-lauroylsarcosine (Sarkosyl) 0.5% (w/v) at room temperature. The mixture was vortexed every 5 min for 10 sec to differentially solubilise the cytoplasmic membrane proteins.

The samples were again centrifuged to pellet the outer-membrane proteins at 42000 rpm ($100,000 \times g$) for 1 h. The supernate contained the sarkosyl-soluble inner-membrane proteins and the gelatinous pellet contained the sarkosyl-insoluble outer-membrane proteins. The pellet was then resuspended in envelope buffer and further centrifugation performed as previously described at 42000 rpm for 1 h. The outer membrane-enriched fractions were resuspended in approximately 1 ml (final volume depends on size of pellet) of 10 mM sodium phosphate (pH 7.2). The final solutions were stored at -70°C for further use, and 50 μl volumes of outer membrane transferred to eppendorf tubes used for protein assay.

2.11.2 Expression of recombinant proteins

Overnight cultures (5 ml) of *E. coli* strains (Table 2.1) containing expression constructs were used to inoculate 2 L dimpled flasks containing 500 ml volumes of LB broth with appropriate antibiotics. The cultures were then incubated at 37°C with shaking until an $\text{OD}_{600\text{nm}}$ of 0.7-0.9 was obtained. IPTG (1mM final concentration) was added to the culture and incubated at 37°C on the shaker for a further 3h. The cells were harvested at 4000 rpm ($5000 \times g$) for 20 min in a Sorvall superspeed centrifuge (rotor GS-3). For further processing, the supernate was discarded and the pellet resuspended in 5-10 ml of PBS and then sonicated (next section).

2.11.3 Preparation of bacterial sonicated extract

Sonicated extracts of different bacteria were prepared from 24-72 h broth cultures for *E. coli* or *B. pertussis*, respectively. A purity test was done using Gram-staining of a smear preparation and direct checking by light microscopy. Cells were collected by centrifugation at $5,000 \times g$ for 20 min at 4°C and washed three times with PBS. A thick suspension of bacteria in PBS was lysed by sonication using a Vibra Cell ultrasonic processor (Jencons-PLS, Leighton Buzzard, United Kingdom) for three 60-s bursts with intermittent cooling on ice. The broken-cell suspension was centrifuged at $3,000 \times g$ for 30 min at 4°C , and the supernate was filtered through a 0.2 μm pore size membrane (Sartorius). The protein concentration was determined by modified Lowry procedure (section 2.11.4.1) and adjusted to a desired concentration by adding sterile PBS.

2.11.4 Protein estimation

All of the cell-free extracts, OMP and other bacterial preparations were subjected to protein quantification to measure the protein content and to set the desired concentrations. The most common methods for the colourimetric detection and quantitation of total protein can be divided into two groups based upon the chemistry involved. Protein assay reagents involve either protein-dye binding (coomassie) chemistry or protein-copper chelation chemistry.

2.11.4.1 Lowry (modified) procedure for membrane proteins (Folin-Ciocalteu)

In Lowry protein assay, or Lowry Method, copper (II) ion in alkaline solution reacts with protein to form complexes, which react with the Folin-phenol reagent, a mixture of phosphotungstic acid and phosphomolybdic acid in phenol. Bound reagent is slowly reduced and changes color from yellow to blue and can be detected colourimetrically by absorbance at 650 nm.

To make the working solution, 100 parts of solution A [2% (w/v) Na_2CO_3 , 0.4% (w/v) NaOH, 0.16% (w/v) sodium tartarate, 1% (w/v) SDS and distilled water] were mixed with 1 part of reagent B [4% (w/v) CuSO_4 and distilled water] to form reagent C, the alkaline copper reagent. Sample volumes of 1 ml (50 μl sample + 950 μl dH_2O) containing 10-100 μg of protein, along with the protein standard BSA (Bovine Serum Albumin) ranging from 20-500 $\mu\text{g}/\text{ml}$ were added to 3 ml of reagent C and incubated at room temperature for 60 min.

The samples were then vigorously mixed with 0.3 ml of diluted Folin-Ciocalteu reagent (1vol:1vol) with distilled water and incubated for 45 min at room temperature. The absorbance was determined at 630 nm on a spectrometer using blanks made up as above except that the protein was replaced by water.

2.11.4.2 Standard procedure for soluble proteins (Bio-Rad)

The Bradford protein assay is a simple procedure for determination of protein concentrations in solutions that depends upon the change in absorbance of Coomassie Blue G-250 upon binding of protein (Bradford, 1976). This method is not susceptible to interference by a wide variety of chemicals present in samples except at high

concentrations of detergents. Concentrated dye reagent was diluted using 1 part with 4 parts of double distilled water according to the manufacturer's instructions. Dilutions of standard protein (BSA) and unknown samples were prepared in 96-well microtiter plates.

The standard was dissolved in the same buffer as the unknowns. Protein solutions were normally assayed in duplicate or triplicate. 20 µl of each sample solution or standard, containing different protein concentrations, was added into microtiter plate wells. Then, the diluted dye reagent (200µl) was added to each well. The sample and reagent were thoroughly mixed using a multichannel pipette and incubated at room temperature for 15 min (maximum 1h) and absorbance measured at 595nm. Finally, the protein concentration in the test was estimated from the standard curve.

2.11.5 Sample preparation for electrophoresis

10 µl of 6× protein loading dye (Appendix A1.3) was mixed with 50 µl of sample. Samples were stored at -20°C until loading. Samples, except marker, were heated in a boiling water bath for 5 min just before loading into the polyacrylamide gel to denature the proteins. A protein marker (SeeBlue® Plus2, Invitrogen) which consisted of 10 pre-stained protein bands (8 blue and 2 contrasting colours) in the range of 4-250 kDa was used according to the manufacturer's instructions.

2.11.6 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Gel cassette preparation and Mini-PROTEAN® 3 Electrophoresis Module assembly was carried out according to the manufacturer's instructions (Bio-Rad). The resolving gel (Appendix A1.3) containing 10% acrylamide was poured into the gap between two glass plates and 100% ethanol was used as an overlay. The gel was allowed to set for approximately 1 hour in a vertical position at room temperature. The overlay prevents oxygen from diffusing into the gel and inhibiting polymerization. Once the polymerization was completed (30 min), the overlay was poured off and the top of the gel washed several times with deionized water to remove any unpolymerized acrylamide. From the top of the gel, as much fluid as possible was drained and any remaining water removed with the edge of a paper towel. Stacking gel (Appendix A1.3) containing 4% acrylamide in a 1 ml volume was poured on top of the resolving gel and a comb was immediately placed within the stacking gel, which was then allowed to set at room temperature. The comb was

removed and samples were run for 45 min at 200V in 1× electrophoresis buffer (Appendix AI.3.2.1) or until the bromophenol blue reached the bottom of the resolving gel. The gel was removed and stained with Coomassie blue (AI.3.1.4) overnight on a slowly rotating platform for protein visualisation. The stain was replaced with destain solution (Appendix AI.3) until the background was decolourised.

2.11.7 Western blotting protocol and development of blots

The proteins of the sample were separated according to size using SDS-PAGE (section 2.7). Then, the proteins in the gel were transferred to immobilising nitrocellulose membrane (Hybond-C, Amersham) according to the method described by Towbin *et al.* (1979). Briefly, the stacking gel was cropped and the separating gel soaked in electroblotting buffer (Appendix AI.3) for 5 min. The blot was then assembled and placed in the electroblotting apparatus containing electroblotting buffer that was stirred magnetically. The protein was transferred from the gel to the membrane using a constant 100 volts for 1 h or, occasionally, at 20 volts overnight. To prevent overheating, and the consequent formation of air bubbles in the sandwich, transfer was carried out using an ice-pack in the tank.

To visually determine whether the proteins were transferred effectively, and to locate molecular-weight markers, the proteins on the membrane were visualised by staining in Ponceau S solution (Sigma, 0.1% w/v Ponceau S in 5% (v/v) acetic acid) for 1-2 min. Background was removed by destaining in water for 2 min and the positions of the molecular weight markers were marked with indelible ink. The membrane was then destained in PBS for 10 min and placed in a heat-sealable plastic bag to which was added 0.1 ml of blocking buffer (Appendix AI.3) per square centimetre of filter. The membrane was kept for 1 h at room temperature with gentle agitation on a platform shaker in order to prevent non-specific protein interaction between the membrane and the antibody. The plastic bag was then opened and the blocking solution discarded. The primary polyclonal antibody was then diluted appropriately in blocking buffer (1:250-1000) and incubated with the membrane for 1 h at room temperature with gentle agitation. The membrane was then washed for 15 min in two changes of PBS at room temperature. Horseradish peroxidase-conjugated secondary monoclonal antibody (anti-rabbit or anti-mouse IgG peroxidase conjugate as appropriate (cat. No. A 0168, Sigma) was diluted appropriately (1:10000) in blocking buffer, added to the membrane and incubated for 1 h at room temperature. Then, the membrane was washed twice in two changes of fresh PBS for 15 min at room temperature. The membrane was then immersed in freshly prepared 3, 3`

diaminobenzidine (DAB) (Sigma) (Appendix I) solution (0.05% w/v in PBS) for approximately 1-2 min and the staining reaction was stopped by washing the membrane in distilled water.

The primary antibody used for these blots was a rabbit polyclonal anti-BapC serum, which had been prepared (Bokhari, PhD thesis 2002) against the purified, His-tagged recombinant truncated BapC expressed in *E. coli* expression strain M15 from the construct pQE60 (Blackburn, 2000). The BapC fragment had been further purified by SDS-PAGE and transferred by blotting onto a nitrocellulose membrane. Then, the BapC band was excised from the membrane and an extract sent to Dr. D. Xing, NIBSC, London to raise a rabbit antiserum.

To minimize the effect of cross-reacting antibodies in immunoblotting of recombinant BapC protein expressed by *E. coli* (section 3.10), an absorbed BapC serum was utilised. *B. pertussis* BP Taberman (BapC⁻) and *E. coli* M15 (pREP4) strains were grown overnight in appropriate liquid media and antibiotics. The cultures were then centrifuged and washed 3 times with saline and the resultant suspensions were adjusted to OD_{600nm} of 0.8-1.0. Cells were sonicated for a few min and the resultant sonicates used to absorb the anti-BapC serum. The anti-BapC serum was mixed twice, with an equal volume of sonicates for 30 min followed by centrifugation steps.

2.11.8 Protein identification by mass spectrometry

Selected bands were manually excised from an SDS-PAGE gel and placed in a 1.5 ml eppendorf tube for transfer to the Sir Henry Wellcome Functional Genomics Laboratory at the University of Glasgow. Mass spectrometry analysis was performed by Dr. R. Burchmore following standard protocols for LC-MS/MS (Mutapi *et al.*, 2005). The peptide mass fingerprint obtained by MS was subjected to a database search by pasting the peptide masses into a protein database (MASCOT[®], Matrix Science), which uses a probability-based scoring system to define a significant match with known protein sequences.

2.12 *In vitro* tests

2.12.1 Effect of antimicrobial peptides

2.12.1.1 Radial diffusion assay

This method of radial diffusion (Lehrer *et al.*, 1991) was used to determine the effect of antimicrobial peptides on *B. pertussis* strains. *B. pertussis* strains were grown on Bordet-Gengou agar medium supplemented with 12% (v/v) horse blood as described previously. The bacteria were harvested in modified Stainer Scholte (Appendix AI.1) broth to an optical density at 600 nm of approximately 0.2, and 0.2 ml of this suspension was added to 10 ml of molten (52°C) 1% agarose (type I; low electroendosmosis; catalogue no. A 6013, Sigma) in modified SS broth containing supplements and 0.15% bovine serum albumin (Sigma).

The agarose was dispersed into petri dishes and was allowed to harden. Holes (3 mm in diameter) were made with an aspirator punch and 5 µl of peptide (Sigma) serially diluted in sterile, filtered distilled water was placed therein. After a 4-h incubation at room temperature to allow for diffusion, a 10-ml overlay of SS-agarose without bacteria was added. The resultant zones of inhibition were read and measured 24 to 48-h later with a metric scale under a stereomicroscope. The Student *t* test was employed to analyse the data.

2.12.1.2 Microtiter plate assay

The C-50%, that is the concentration of peptide according to the method of Fernandez and Weiss (1996) for killing or inhibiting the growth of 50% of the organisms, was determined as follows. Serial dilutions of the different antimicrobial peptides were prepared in 100 µl of RPMI medium in a microtiter plate. 10 µl of *B. pertussis* in modified SS-broth (see above) was added to each well to a final concentration of approximately 2×10^6 CFU/ml. Then, after a 2-h incubation at 37°C, 100 µl from each well was diluted in 900 µl of SS-broth and the number of survivors were ascertained by plating out 10-fold serial dilutions on Bordet-Gengou agar. As a control, bacteria were similarly added to wells containing RPMI medium but no peptides. The C-50% was determined and adjusted according to the purity of the peptide stated by the manufacturer.

2.12.2 Agglutination test

This test was carried out to detect surface-exposed BapC in *B. pertussis* and *E. coli* strains using the rabbit anti-BapC serum used previously (section 2.11.7). The BapC mutant was used as a negative control in the agglutination tests. An antibody raised against whole *B. pertussis* cells, available from departmental stocks, was also used in the study. *B. pertussis* strains were collected from BG plates that had been incubated for 48-72 h at 37°C. *E. coli* strains were collected from LB plates that had been incubated for 18-24 h at 37°C. One ml of a thick bacterial suspension was obtained by centrifuging ~7 ml volume of culture containing $\sim 2 \times 10^9$ CFU/ml of the various *B. pertussis* strains and the pellets were re-suspended in 1 ml of PBS. A 100 µl volume from the thick suspension was added to individual wells in a 96-well U-shaped plate (Greiner, UK) in triplicate. 50 µl volumes of 10-fold dilution of antisera were thereafter added to each well. The samples were then mixed thoroughly in each well with a multichannel pipette and plates placed on a rotating shaker at room temperature for 1h. Plates were then incubated at 37°C for 3-4 h and finally left at 4°C for 24-48 h. Agglutination or button formation at the bottom of each well, and also the type of agglutination, was recorded.

2.12.3 Serum killing assay

2.12.3.1 Collection of normal human serum

Serum was obtained from the blood samples of 8-10 volunteers from the Division of Infection and Immunity who were not actively engaged in working with *B. pertussis*. After collection, the blood samples were allowed to clot for 1 h at 37°C. They were then placed on ice for 1-2 h to retract and the clear serum was collected after centrifugation at 10000×g for 5 min without disturbing the clot. The serum was centrifuged again to remove any remaining particles and the supernate was collected. The individual sera were pooled, distributed into 2 ml aliquots and stored frozen at -70°C.

2.12.3.2 Bactericidal assays with normal human serum

B. pertussis cells were harvested from BG agar plates incubated at 37°C for 24-48 h and suspended at $\sim 2 \times 10^9$ CFU/ml by comparison with the opacity rod (5th International Reference of Opacity, Perkins *et al.*, 1973), in pre-warmed (37°C) Stainer and Scholte (SS-X) medium. A volume of 100 µl of bacterial suspension was transferred to an eppendorf

tube containing 350 µl of SS-X medium and 50 µl of normal or heat-inactivated (30 min at 56°C) control serum and the contents mixed.

The tubes were incubated at 37°C in a water bath for 45 min. The tubes were then shifted to ice for 5 min to stop the complement reaction. A 20-µl aliquot of the mixture was added to 180 µl of phosphate-buffered saline (PBS) (pH 7.4) with 10 mM EDTA (inactivates complement activity). Ten-fold serial dilutions were performed in SS-X medium. The dilutions were then plated in duplicate on BG agar, and incubated at 37°C.

Bacterial colony forming units were counted after 3-4 days. Survival in normal human serum was calculated as a percentage of the number obtained from the SS medium control (non-killing control without serum). Heat-inactivated (56°C for 30 min) serum also acted as a control in case of an apparent reduction in viable count due to presence of agglutinating antibodies. Percentage killing was calculated by comparison with 100% survival of the SS medium control. Statistical analysis was performed using Student's *t*-test.

2.13 *In vivo* test

2.13.1 Competition assay for mouse virulence of *B. pertussis* strains

In competition assays, the animal is infected with a mixture of mutant and wild type bacteria. After giving the bacteria time to establish themselves, samples can be taken from various parts of the animal, and the ratio of mutant to wild type is determined. If the ratio of the mutant to wild type is the same as in the infecting dose, the mutation had no detectable effect on virulence but, if the wild type outcompetes the mutant, the mutation clearly had a negative effect. In other words, disappearance of the mutant after a certain period of time indicates that, in the animal, the mutant was not able to compete successfully with the wild type.

In this study, the *in vivo* competition assay was done by growing the strains of *B. pertussis* to be compared on BG plates containing appropriate antibiotics for 3 days in humidified conditions at 37°C. Lawn cultures were then made under the same conditions, with antibiotics, for a further 24h. The resultant growth was suspended in 1% (w/v) casamino

acids solution (CAA) (Appendix I) and adjusted to 10 opacity units using the 5th International Reference of Opacity (Perkins *et al.*, 1973) i.e. approx. 2×10^9 CFU/ml.

After serial dilution in 1% (w/v) CAA, 100 μ l volumes of 1×10^3 CFU/ml of two strains to be compared were mixed and individual cultures or the mixture were plated in duplicate on BG agar plates with and without appropriate antibiotics. From plates without antibiotics, the total number of colonies was estimated. By appropriate choice of antibiotics, it was possible to estimate the numbers of just one strain in the mixture. By simple subtraction, the number of the other member of the mixture could be determined and then the ratio of strain 1 : strain 2 calculated. This was done as a preliminary test to investigate the growth effect of one strain on another strain. Colonies were counted after 3-4 days and data analysed with Student's *t*-test.

For the animal experiments, *B. pertussis* suspensions were prepared containing 4×10^6 CFU/ml of each strain, either alone or as a two-strain mixture. Thus, a sublethal dose of 50- μ l volume would contain 1×10^5 CFU of each strain. Randomised groups of five female CD1 mice (Harlan Olac, Bicester, Oxfordshire, UK) aged 3 weeks were allowed to acclimatise for 3-4 days. They were divided into different test groups, which were categorised according to experiment number. Each group comprised five mice and, after light halothane anaesthesia, each mouse was instilled intranasally with 50- μ l volumes of prepared suspensions of *B. pertussis* strains.

Two experiments were set up. In experiment I, mice were intranasally instilled with *B. pertussis* strains BP338 (W/T), BP338 (BapC⁻) mixture; BP338, BP338 (BrkA⁻, BapC⁻) mixture. In experiment II, mice were intranasally instilled with *B. pertussis* strains BP338, BP338 (BrkA⁻) mixture; BP338 (BapC⁻), BP338 (BrkA⁻, BapC⁻) mixture; BP338 (BapC⁻), BP347 (Bvg⁻) mixture; BP338 (BapC⁻), BP338 (BrkA⁻) mixture. The mouse virulent strain 18-323 was used as a positive control, in a separate group.

The weight of each group of mice was recorded prior to challenge as well as at regular intervals afterwards. The mice were sacrificed after 7 days and the lungs were removed aseptically into sterile universal bottles. Lungs were homogenised in 10 ml of CAA with a sterile homogeniser (Silverson machines, UK). 100 μ l volumes from undiluted samples and 1:10, 1:100, 1:1000 and 1:10000 dilutions in 1% (w/v) CAA were cultured on duplicated BG agar plates with and without appropriate antibiotics and incubated at 37°C in a humidified box. Colonies were counted after 3-4 days, and the number of colony

forming units per lung was calculated. Nominal values of 100 and 10^7 were given to counts outside the detection limits <100 and $>3 \times 10^6$ per organ, respectively.

The \log_{10} counts were calculated and the means, standard deviation and standard error values of each group were calculated in Excel. The data were analysed by applying the Student's t-test and one way of analysis of variance (ANOVA).

2.14 Sequence analysis tools (bioinformatics)

Three primary sequence databases; GenBank which is maintained by the National Center for Biotechnology Information (NCBI), the Nucleotide Sequence Database maintained by the European Molecular Biology Laboratory (EMBL) and the DNA Databank of Japan (DDBJ) were used to annotate and highlight the important properties of the raw sequence data. Entrez, a WWW-based data retrieval tool (NCBI), was employed to search multiple biological databases and retrieve relevant information including nucleotide, protein sequences and whole genomes. Entrez can be accessed via the NCBI web site at the following URL: <http://www.ncbi.nlm.nih.gov/Entrez/>. Sequence similarity searches of databases were performed to extract the similarities using Basic Local (Linear) Alignment Search Tool (BLAST) and FASTA programs. Information about these extracted sequences can be used to predict the structure or function of the query sequence. BLAST utilizes statistical theory to produce a bit score and expect value (E-value) for each alignment pair (query to hit). The bit score indicates how good the alignment is; the higher the score, the better the alignment. The E-value indicates the statistical significance of a used pairwise alignment and reveals the size of the database and the scoring system. The lower the E-value, the more significant the hit. A sequence alignment that has an E-value of 0.05 means that this similarity has a 5 in 100 (1 in 20) chance of occurring by chance alone. The p value of a similarity score is the high probability of obtaining the score in a chance similarity between two unrelated sequences of similar composition. Low p value corresponds to significant matches that are likely to have real biological significance. In general, the score is calculated by an equation which considers identical or similar residues and any gaps in the aligned sequences using BLOSUM62 to assign a score for aligning any possible pair of residues.

Many proteins are composed of domains in a modular architecture. Pfam and SMART (Simple Modular Architecture Research Tool; <http://smart.embl-heidelberg.de>) were employed to analyze protein domain families. The domain within the query sequence in the SMART program was selected and the BLAST run based on BLOSUM62 matrix, filter

default and E-value 10 by the Bork group's advanced BLAST2 Search Service at EMB. The Pfam database (<http://www.sanger.ac.uk/Software/Pfam>) is a collection of many common protein domains and families, and HMMs (Hidden Markov models).

ProtParam is a tool which allows the computation of various physical and chemical parameters for a given protein stored in [Swiss-Prot or TrEMBL](#) or for a user-entered sequence. The computed parameters include the molecular weight, theoretical pI, amino acid composition, atomic composition, extinction coefficient, estimated half-life, instability index, aliphatic index and grand average of hydropathicity.

3. Results

3.1 Characterisation of *B. pertussis* strains

A number of strains, which were available from previous studies (Blackburn, 2000; Bokhari, 2002), were investigated for their phenotypes and genotypes by growth in the presence of different antibiotics in appropriate concentrations (Table 2.3) by Gram-staining, and by slide agglutination test; respectively. Results are shown in Table 3.1.

All strains listed in Table 3.1 had the expected antibiotic sensitivities / resistances and the properties expected of *B. pertussis* i.e. inability to growth on nutrient agar, Gram-negative, agglutination by anti-whole *B. pertussis* or anti-BapC sera (where appropriate) and production of β -haemolysin on BG sandwich agar, as well as the expected colonial morphology. Some other strains from the departmental culture collection either did not grow or did not have the expected properties and so were not used in the present study. Antibiotic sensitivity showed that BP Tohama W/T and BP Taberman W/T did not grow, as expected, in the presence of Amp, Km, Nal or Sm. Slide agglutination tests demonstrated strong agglutination reactions with both anti-whole pertussis and anti-BapC sera, suggesting the presence of expressed BapC protein on the surface of these organisms. However, with *B. pertussis* Tohama II, a very weak agglutination was observed with anti-BapC sera, and no agglutination was detected with *B. pertussis* Taberman II, suggesting lack of BapC expression in these mutants. The weak reaction with BP Tohama (BapC⁻), could possibly have been due to a cross-reaction with other surface components or due to a large amount of bacteria loaded on the agglutination test. Strains BP Tohama I and BP Taberman I, parents of the BapC mutants, which had been used to construct the original BapC mutant strains, appeared to be authentic in antibiotic sensitivities since they were spontaneous streptomycin-resistant derivatives of the original wild-type strains which possessed nalidixic acid resistance. BP Tohama II (BapC⁻) and BP Taberman II (BapC⁻)

strains were found to have the same antibiotic resistances (Sm^{R} , Nal^{R}) as their parents but also showed kanamycin resistance, since a kanamycin resistance cassette had been inserted into the *bapC* gene using homologous recombinant technology to make BapC mutant strains. BP338 and BP338 (BrkA^-) (2041 Weiss) strains showed the expected phenotype with regard to antibiotic sensitivities, reaction to anti-BapC and anti-whole pertussis sera and other aspects such as the size of the amplified *bapC* fragment in PCR reaction. These strains were used, in this study, to make single (BapC^-) and double (BrkA^- , BapC^-) mutants, respectively). The presence of *bapC* gene of the expected size in BP338 and BP338 (BrkA^-) was confirmed, making them suitable for *bapC* mutant construction in the same genetic background (see next section).

Table 3.1 Antibiotic sensitivities and other features of *B. pertussis* strains available at the start of this study

Strain		Antibiotic sensitivity / other properties								PCR product*	
		Amp	Km	Nal	Sm	N.A.	Gram reaction	S.A.T. 1	S.A.T. 2		β-h.
1	BP Tohama (W/T)	S	S	S	S	–	-ve	+	+	+	2.2 kbp
2	BP Tohama parent of BapC mutant (Tohama I) Sm ^R /Nal ^R	S	S	R	R	–	-ve	+	+	+	2.2 kbp
3	BP Tohama (BapC ⁻) (Tohama II) Sm ^R /Nal ^R /Km ^R	S	R	R	R	–	-ve	+	±	+	3.5 kbp
4	BP338 (Weiss) Nal ^R	S	S	R	S	–	-ve	+	+	+	2.2 kbp
5	BP338 (BrkA ⁻) (2041 Weiss) Nal ^R /Km ^R	S	R	R	S	–	-ve	+	+	+	2.2 kbp
6	BP Taberman (L1) (W/T)	S	S	S	S	–	-ve	+	+	+	2.2 kbp
7	BP Taberman parent of BapC mutant (Taberman I) Sm ^R /Nal ^R	S	S	R	R	–	-ve	+	+	+	2.2 kbp
8	BP Taberman (BapC ⁻) (Taberman II) Sm ^R /Nal ^R /Km ^R	S	R	R	R	–	-ve	+	-	+	3.5 kbp

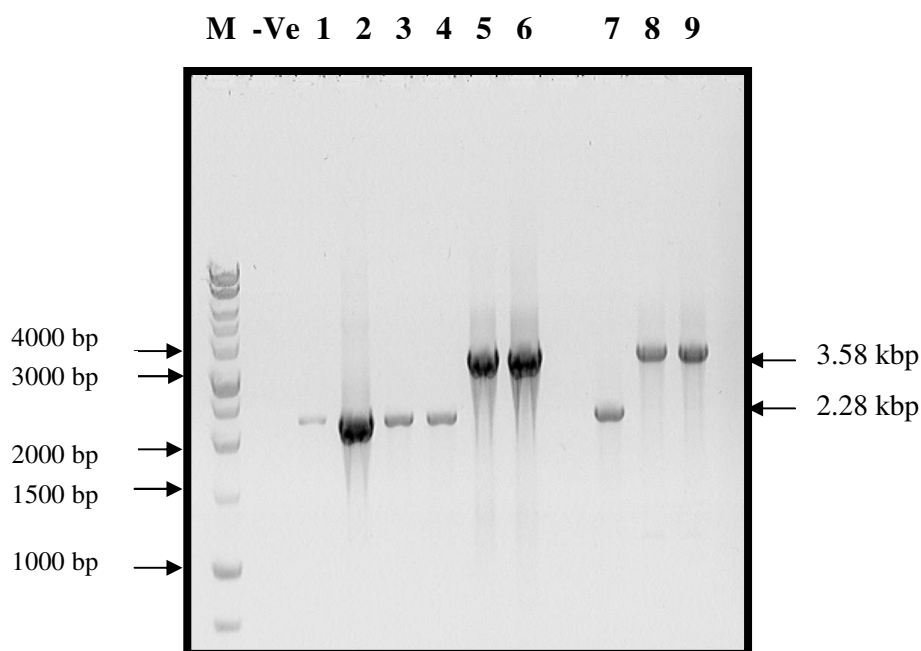
Amp: Ampicillin**Km:** Kanamycin**Nal:** Nalidixic Acid**Sm:** Streptomycin**R:** Resistance**S:** Sensitive**N.A:** Growth on Nutrient Agar**β-h:** β-haemolysis on BG sandwich agar**S.A.T1:** Slide Agglutination Test with rabbit polyclonal anti-whole *B. pertussis* serum**S.A.T2:** Slide Agglutination Test with rabbit polyclonal anti-BapC serum**W/T:** Wild Type

* With primers BAP5F and BAP5R (Table 2.5)

3.2 Confirmation of the *bapC* genotype of the *B. pertussis* strains

PCR reactions with genomic DNA from the wild-type and *bapC* mutant strains of *B. pertussis*, described in Table 3.1, showed the expected sizes of *bapC* (2.2 kbp) and the *bapC* gene with the inserted Km resistance cassette (3.5 kbp), respectively (Fig. 3.1 and Table 3.1). PCR was carried out using the *bapC* specific primers (Table 2.5), *bapC* forward (BAP5F) and *bapC* reverse (BAP5R), which amplify most of the full length *bapC* gene from nucleotide position 728 to 2960 (starting just downstream from the first previously predicted start codon site) (Appendix II.). The PCR results using genomic DNA extracted from *B. pertussis* Taberman I strain and its *bapC* defective mutant (Taberman II), as well as *B. pertussis* Tohama I and its *bapC* defective mutant (Tohama II), indicated that *bapC::Km^r* is present in the chromosome of the mutant strains. This is clear evidence that *bapC* gene has been replaced by the mutated allele, *bapC::Km^r*. The difference between the parent strains and the *bapC* mutant strains is expected from the insertion of the 1.3 kbp kanamycin resistance cassette. The PCR reaction with the BP338 (BrkA⁻) genomic DNA also confirmed *bapC* of the expected size, 2.2 kbp, indicating the presence of the unaltered *bapC* gene in this strain.

Figure 3.1 Agarose (0.8%) gel showing the PCR amplimers derived from the *bapC* gene in *B. pertussis* strains



M = DNA marker

-Ve = Negative

1 = BP338 (Weiss)

2 = BP338 (BrkA⁻) (2041 Weiss)

3 = BP Tohama (W/T)

4 = BP Tohama I (parent of BapC mutant)

5 = BP Tohama II (BapC⁻)

6 = BP Tohama II (BapC⁻)

7 = BP Taberman (parent of BapC mutant)

8 = BP Taberman II (BapC⁻)

9 = BP Taberman II (BapC⁻)

PCR was performed on chromosomal DNA (extracted as described in section 2.2.1) using primers BAP5F and BAP5R (Table 2.5) which amplify most of the *bapC* gene.

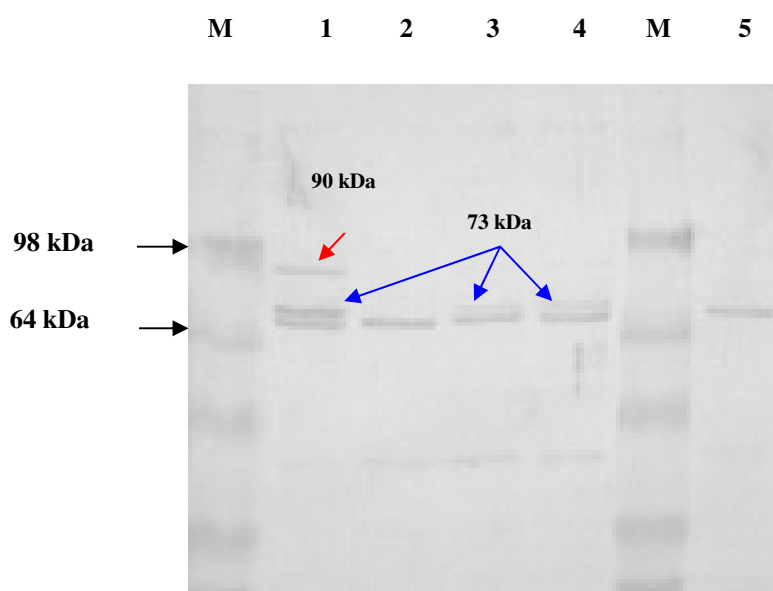
3.3 Characterization of BapC protein in *B. pertussis* strains by immunoblotting

Immunoblotting experiments with a rabbit anti-BapC serum were done with *B. pertussis* strains that were expected to be expressing the BapC protein {BP Taberman and BP Tohama wild-type strains, BP338 and BP338 (BrkA⁻)} or not expressing the protein (i.e., when grown in modulating conditions with MgSO₄; in BP Taberman II and BP Tohama II BapC⁻ mutants) or BP347 (Bvg⁻ strain). The results are shown in Figures 3.2 and 3.3.

As can be seen from Figures 3.2 and 3.3, a band of approximately 70 kDa appeared to be expressed in all strains, whether grown under modulating or non-modulating conditions and this presumably represents a non-Bvg-regulated protein that may be antigenically related to BapC and other autotransporter proteins. It would have been interesting to have identified this protein by N-terminal sequencing or mass spectrometry. The 73-kDa protein (which is the expected size of the mature form of BrkA) in Figures 3.2 & 3.3 is presumably the processed form of BrkA protein that also appears to cross-react with the anti-BapC serum and this was not present in modulating conditions nor in the *brkA* mutant (BP2041). There was an interesting and potentially important difference between Taberman and Tohama wild-type and BapC mutants, where the 73-kDa protein seemed to be fainter in both Tohama II (BapC⁻) and Taberman II (BapC⁻) mutant strains compared to their wild-type strains, suggesting that a lack of BapC influenced the amount of BrkA made by the cells. A band at ~90 kDa is presumed to represent some form of BapC since it was present in the wild-type *B. pertussis* strains; BP Taberman (W/T) (lane 1, Fig. 3.2), BP Tohama (W/T) and BP338 (lanes 1 and 3, respectively, Fig. 3.3); and also in BP338 (BrkA⁻) (BP2041) (lane 5, Fig. 3.3) but absent in the *bapC* mutants: BP Taberman II (lanes 3 and 4, Fig. 3.2), and BP Tohama II (BapC⁻) (lane 7, Fig. 3.3). The absence of this 90 kDa band in BP338 (Bvg⁻) (BP347) and in all strains grown under modulating conditions again indicated that the BapC protein is a *bvg*-regulated protein.

Figure 3.2 Immunoblotting with anti-BapC serum of whole cells of *B. pertussis* Taberman strains grown in modulating and non-modulating conditions.

Equal amounts of the protein were subjected to SDS-PAGE (10%), and then blotted to Hybond-C membrane and probed with anti-BapC serum.



M= Marker

1= BP Taberman (W/T)

2= BP Taberman (W/T) + MgSO₄

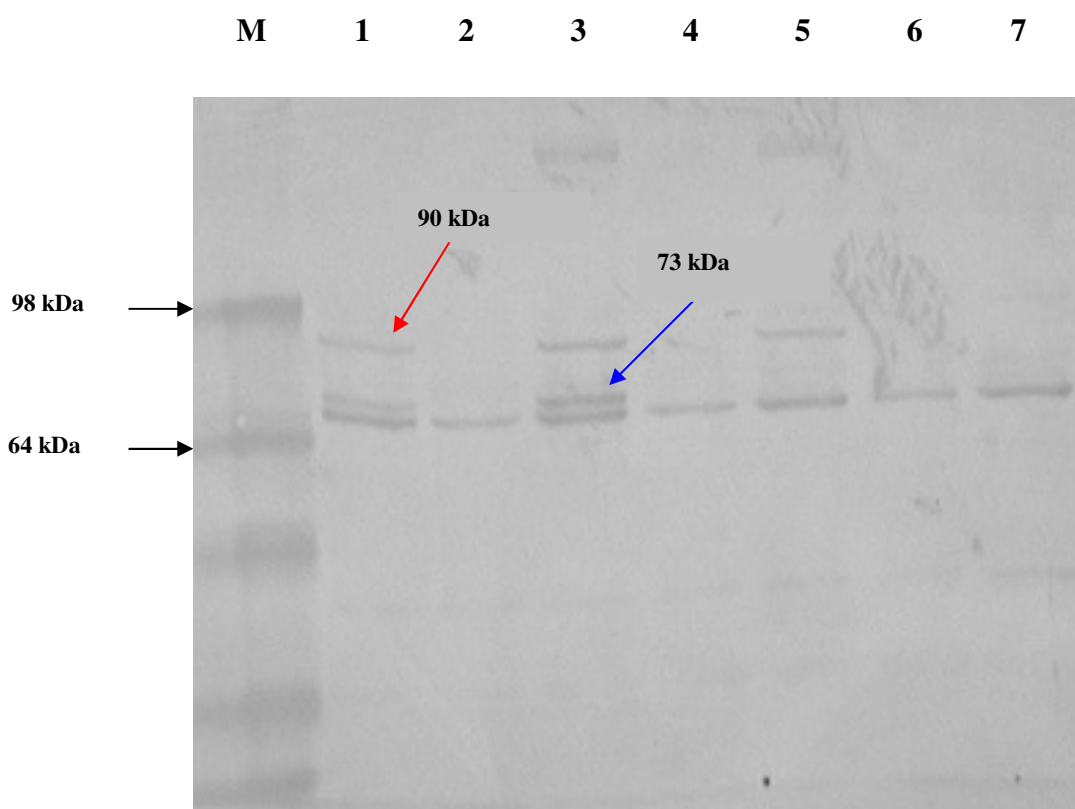
3= BP Taberman II (BapC⁻)

4= BP Taberman II (BapC⁻)

5= BP338 (Bvg⁻) (BP347)

Figure 3.3 Immunoblotting with anti-BapC serum of *B. pertussis* Tohama strains grown in modulating and non-modulating conditions

Equal amounts of the protein were subjected to SDS-PAGE (10%), and then blotted to Hybond-C membrane and probed with anti-BapC serum.



M= Marker

1= BP Tohama (W/T)

2= BP Tohama (W/T) + MgSO₄

3= BP338

4= BP338 + MgSO₄

5= BP338 (BrkA⁻) (BP2041)

6= BP338 (BrkA⁻) + MgSO₄

7= BP Tohama II (BapC⁻)

3.4 Localisation of BapC protein in outer-membrane preparations of *B. pertussis* strains

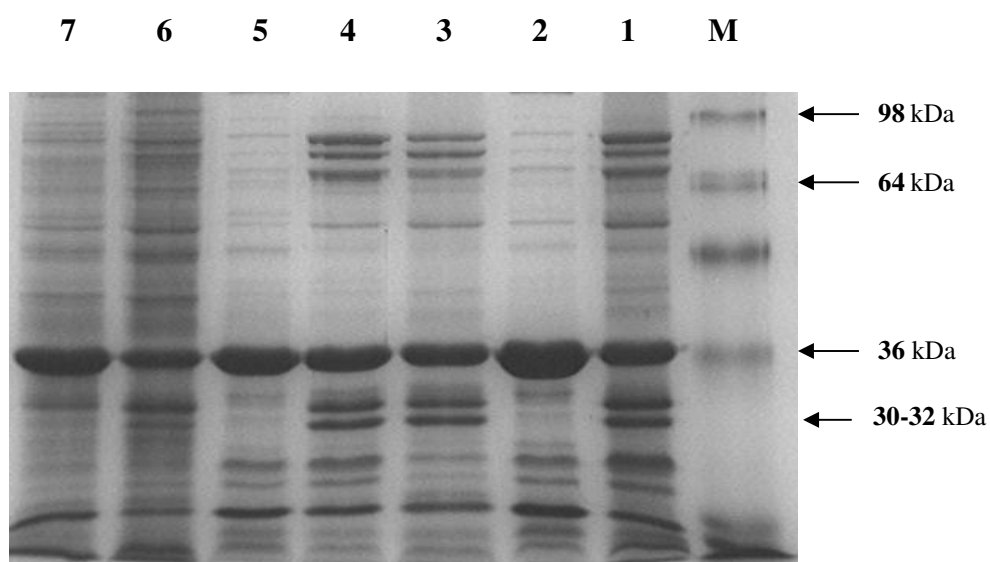
This experiment was intended to give information on the nature and localisation of the BapC protein in the outer membrane fraction of *B. pertussis* cells. Figures 3.4 and 3.5 show the protein profiles and immunoblots with rabbit anti-BapC serum, respectively, of *B. pertussis* strains grown in modulating (nicotinic acid, 16mM) and non-modulating conditions. Major differences in Fig. 3.4 are in the 30-32 kDa and ~90 kDa regions. These bands were not found in the modulated or Bvg⁻ strain, BP347. The 30-32 kDa bands correspond in size to the β -domains (see Introduction, section 1.6.5) of the various autotransporters of *B. pertussis* and presumably, therefore, could represent these fragments of the autotransporters left in the outer membrane after cleavage and release of the passenger domains. They are still present in the BP Taberman (BapC⁻) and BP338 (BrkA⁻) strains that, although each lacking one autotransporter, will produce the other autotransporter proteins.

The immunoblots of the outer-membrane preparations gave somewhat different patterns to those obtained with whole cells (Figs 3.2, 3.3). In Fig. 3.5, prominent differences were observed in the ~98 kDa and ~70 kDa regions. The band at ~98 kDa in Fig. 3.5 is possibly the BapC protein as it was present in BP Taberman (W/T), BP338 and faintly in BP338 (BrkA⁻) (although the amount of protein in this sample appears to be low, see Fig. 3.4), but not found in BP Tohama II or in the strains grown in modulated conditions (nicotinic acid, 16mM) or in the Bvg⁻ strain, BP347, which again confirmed the regulation of BapC via *bvg*-regulation system. This band runs at a higher molecular weight than the predicted size of the processed form of BapC i.e. 71 kDa, and it may be the apparent size of the unprocessed form of BapC after the cleavage of a signal sequence which, calculated using the ProtParam tool, is about 100 kDa. Alternatively, the protein runs anomalously on SDS-PAGE. Such behaviour has been previously described for other autotransporter proteins

such as mature pertactin, which has calculated molecular weight of 61 kDa but runs at 69 kDa on SDS-PAGE. The inner-membrane fractions of BP Taberman showed a similar protein profiles to the *bapC* derivative (not shown). The band at ~64 kDa in Fig. 3.5 possibly corresponds to the processed form of BrkA protein (73 kDa) since it was not present in BP338 (BrkA⁻), the Bvg⁻ strain, BP347 or in the strains grown in modulated conditions. These observations also confirm the regulation of BrkA protein by the BvgAS two-component regulatory system. This pattern was found to be similar to that in the whole cells immunoblots (Figs. 3.2 and 3.3) in that 73 kDa was present in the *B. pertussis* wild-type and their BapC mutant strains, whereas it was missing in the BrkA⁻ strain. However, the apparent size differences are difficult to explain. The identity of the other two prominent bands in lanes 1, 3, 4, and 6 in the 64-98 kDa region is unknown. However, they appear to be Bvg-regulated, are abundant in the outer-membrane preparations and cross-react with the anti-BapC serum. It may be that these represent other antigenically-related autotransporter proteins that are more abundant than BapC and BrkA. For example, the lower band could represent the mature form of pertactin (69 kDa) and the upper band could represent mature Vag-8 (95 kDa) or unprocessed form of TcfA (90 kDa) but further work, e.g. by N-terminal sequencing or peptide finger printing, would be required to confirm their identities. It was also curious that the anti-BapC serum did not react with the 30-32 kDa bands even though it was raised against a recombinant protein fragment of BapC that contained the β -domain.

Figure 3.4 Outer-membrane protein profiles of *B. pertussis* strains grown in modulating and non-modulating conditions

Protein profiles were obtained by SDS-PAGE in gel containing 10% acrylamide using equal amounts of the protein.



M = marker

1 = BP Taberman Wild Type (W/T)

2 = BP Taberman Wild Type (W/T) + Nicotinic acid

3 = BP Taberman II (BapC⁻)

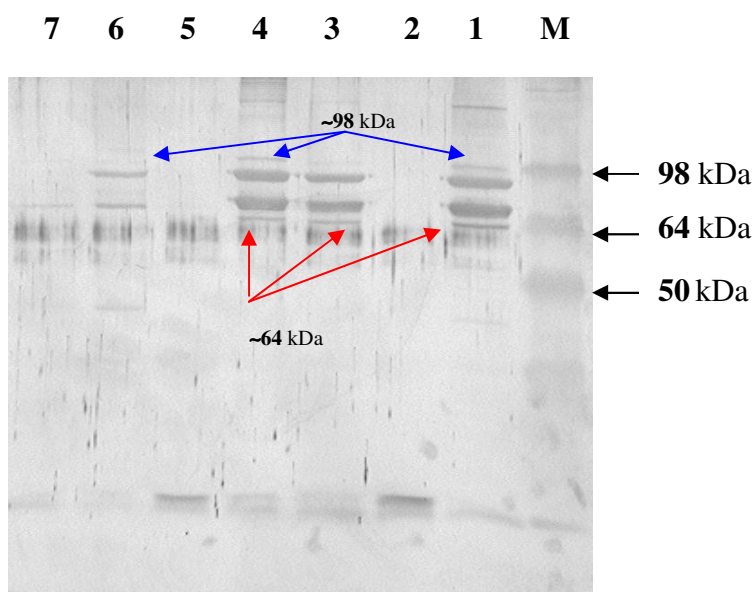
4 = BP338

5 = BP338 + Nicotinic acid

6 = BP338 (BrkA⁻)(BP2041)

7 = BP338 (Bvg⁻)(BP347)

Figure 3.5 Immunoblotting with anti-BapC serum of outer-membrane preparations of *B. pertussis* strains grown in modulating and non-modulating conditions.



M = marker

1 = BP Taberman (W/T)

2 = BP Taberman (W/T) + Nicotinic acid

3 = BP Taberman II (BapC⁻)

4 = BP338

5 = BP338 + Nicotinic acid

6 = BP338 (BrkA⁻)(BP2041)

7 = BP338 (Bvg⁻)(BP347)

Equal amounts of the protein were subjected to SDS-PAGE (10%), and then blotted to Hybond-C membrane and probed with anti-BapC serum.

3.5 Localisation of BapC in *B. pertussis* strains

In an attempt to investigate the expression of BapC protein in *B. pertussis* strains, an agglutination procedure was carried out using the rabbit polyclonal anti-BapC serum absorbed with a *B. pertussis* BapC mutant strain (section 2.12.2) to reduce the cross-reaction with other *B. pertussis* antigens that was evident from the immunoblots. The agglutination test was performed in 96-well u-shaped microplates using bacterial cells scraped from BG plates and suspended in PBS and the suspension standardized at OD₆₀₀ ~ 0.8. The polyclonal anti-BapC serum strongly agglutinated the *B. pertussis* strains expressing BapC protein.

Table 3.2 shows the agglutination reaction of the different *B. pertussis* strains with the rabbit polyclonal anti-whole cell and the (absorbed) anti-BapC sera and these results largely agree with those shown for the unabsorbed anti-BapC serum in Table 3.1. These strains were strongly agglutinated by the anti-whole cell pertussis serum, confirming the reliability of strains used in the study. The absorbed polyclonal anti-BapC serum strongly agglutinated *B. pertussis* strains which should have been expressing BapC such as; BP Taberman (W/T), BP338 and BP338 (BrkA⁻). However, there was only a weak agglutination with the *B. pertussis* BP Tohama wild-type strain. Much less agglutination was seen with strains which should not express BapC [(BP Tohama (BapC⁻) or Bvg⁻ (BP347)], and not at all with BP Taberman (BapC⁻) or any of the strains grown in the presence of a high concentration of MgSO₄ (Bvg⁻ phase). These results also confirmed that expression of the BapC protein is controlled by the BvgAS two-component regulatory system since it was not detected by anti-BapC serum under modulating conditions. Another important finding was that the *B. pertussis* BP338 (BrkA⁻) strain expressed the BapC protein because it was strongly agglutinated by the anti-BapC serum.

Table 3.2 Agglutination of *B. pertussis* strains grown in modulating (+MgSO₄) and non-modulating conditions

Strains	Agglutination with anti whole-cell <i>B. pertussis</i> serum	Agglutination with anti-BapC serum *	Bacteria alone (Self-agglutination control)
<i>B. pertussis</i> Taberman W/T	++++	++++	–
<i>B. pertussis</i> Taberman W/T (+ MgSO ₄)	+++	–	–
<i>B. pertussis</i> Taberman II (BapC)	+++	–	–
<i>B. pertussis</i> Tohama W/T	+++	±?	–
<i>B. pertussis</i> Tohama W/T (+ MgSO ₄)	+++	–	–
<i>B. pertussis</i> 338 Weiss	++++	++++	–
<i>B. pertussis</i> 338 Weiss (+ MgSO ₄)	+++	–	–
<i>B. pertussis</i> 338 (BrkA [–]) (2041 Weiss)	++++	++++	–
<i>B. pertussis</i> 338 (BrkA [–]) (2041 Weiss) (+ MgSO ₄)	+++	–	–
<i>B. pertussis</i> Tohama II (BapC)	+++	+	–
<i>B. pertussis</i> 338 (Bvg [–]) (BP347)	+++	+	–

- = No agglutination

+ = Weak agglutination

++ = Moderate agglutination

+++ = Strong agglutination

++++ = Very strong agglutination

(+ MgSO₄) = denotes growth in modulating (Bvg[–]) conditions

* The serum was absorbed with whole cells of *B. pertussis* BP Taberman II strain to remove antibodies to any other surface proteins.

3.6 Serum killing assays of *B. pertussis* strains

The serum killing assays were carried out to investigate whether BapC due to its structural homology with the autotransporter protein BrkA, could confer a serum resistance phenotype on *B. pertussis*. Previous work by Bokhari (PhD thesis, 2002) had suggested that the BapC protein of *B. pertussis*, like BrkA, could confer resistance to killing by normal human serum. The main objective of the present work was to confirm and extend these findings and to determine the relative contribution of the two proteins to serum resistance. The first step was to characterize the serum sensitivity of the *B. pertussis* and recombinant *E. coli* strains that were available at the start of the present study.

All of the serum killing assays in the present investigation were performed using pooled normal human serum (section 2.12.3.1) obtained from volunteers with no recent history of pertussis. The survival was calculated from the number of survivors from the normal human serum as a percentage of the number of survivors from heat-inactivated serum. The heat treatment (56°C for 30 min) destroys the inherent complement activity of the serum. A preliminary comparison of the number of survivors in PBS controls (i.e. no added serum) and in heat-inactivated serum did not show any significant difference over the incubation period. These observations suggested that any decrease in bacterial counts due to agglutination by any antibodies present in the normal human serum, rather than by killing, was negligible. Different normal human serum dilutions (10%, 20%, 30%, 50% and neat) in a time-course study (exposure for 30, 45, 90 and 120 min) were performed to determine the optimal time of exposure to normal human serum. The time-course of killing different *B. pertussis* strains by 10% normal human serum is shown in Fig. 3.6a. From these time-course results, an incubation time of 45 min was selected for further serum killing assays as there was little change thereafter in the number of CFU of *B. pertussis* recovered from the normal serum. In these experiments, BP338 and its BrkA⁻ mutant (BP2041) were used as controls.

Figure 3.6b shows a comparison of the serum sensitivity of some of the various *B. pertussis* strains available at the start of this study, when exposed to normal human serum (NHS) for 45 min. *B. pertussis* BP338 and *B. pertussis* Taberman (wild type) were the most resistant to killing, showing some survival even in neat serum after 45 min. Moreover, *B. pertussis* Tohama derivative, BP338, seemed to be inherently more resistant compared to the *B. pertussis* Tohama wild type. The Tohama wild type strain was somewhat more sensitive but still showed some (i.e. 5%) survival in neat serum. *B. pertussis* BP2041, the BrkA mutant, was much more sensitive than its parent strain BP338, with 10% survival in 1/3 dilution of serum and no detectable survival in neat serum. The difference in serum sensitivity between the *brkA* mutant and its parent strain BP338 was found to be similar, but not as great, as that reported by Fernandez and Weiss (1994). The BapC mutant strains, BP Tohama II and BP Taberman II, constructed by Bokhari (2002) were more sensitive than their parents and showed a similarly sensitivity to *B. pertussis* BP2041.

Although in all experiments pooled human serum was utilised, the activity of the complement proteins which are the key players in killing of *B. pertussis*, could be affected by the preparation steps involved and during storage of the serum. An experiment was designed to assess serum killing using de-complemented serum, by heating it to 56°C for 30 min, and adding a prepared source of complement (guinea pig serum, Sigma). The guinea pig serum was expected to be free of antibodies against *B. pertussis*, whereas the heat-inactivated human serum could possibly act as a source of cross-reacting antibodies. 5% v/v guinea pig serum, as a source of complement, was added to the 10% v/v heat-inactivated human serum. As expected, *B. pertussis* BP338 was found to be more resistant compared to its *brkA* mutant strain, BP2041 (data not shown). Therefore, the data obtained from the use of an external source of complement added to the de-complemented human serum supported the previous findings. It was also concluded that the activity of the complement proteins of the human serum preparation was preserved.

Figure 3.6a. Time course of killing of *B. pertussis* strains by 10% normal human serum (NHS)

The results are the means of three separate experiments and bars indicate the standard deviations.

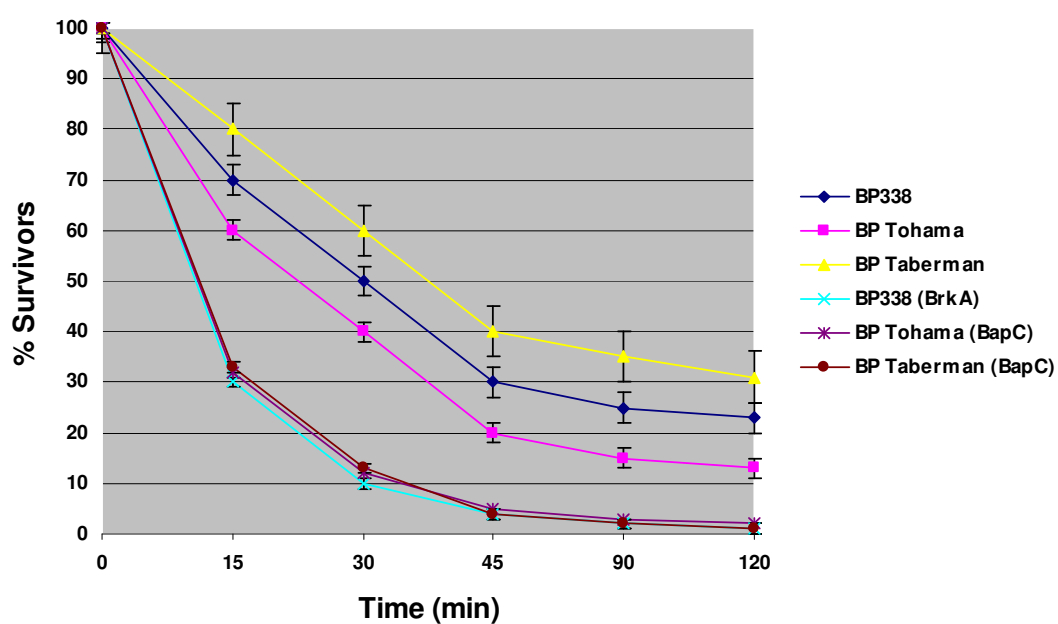
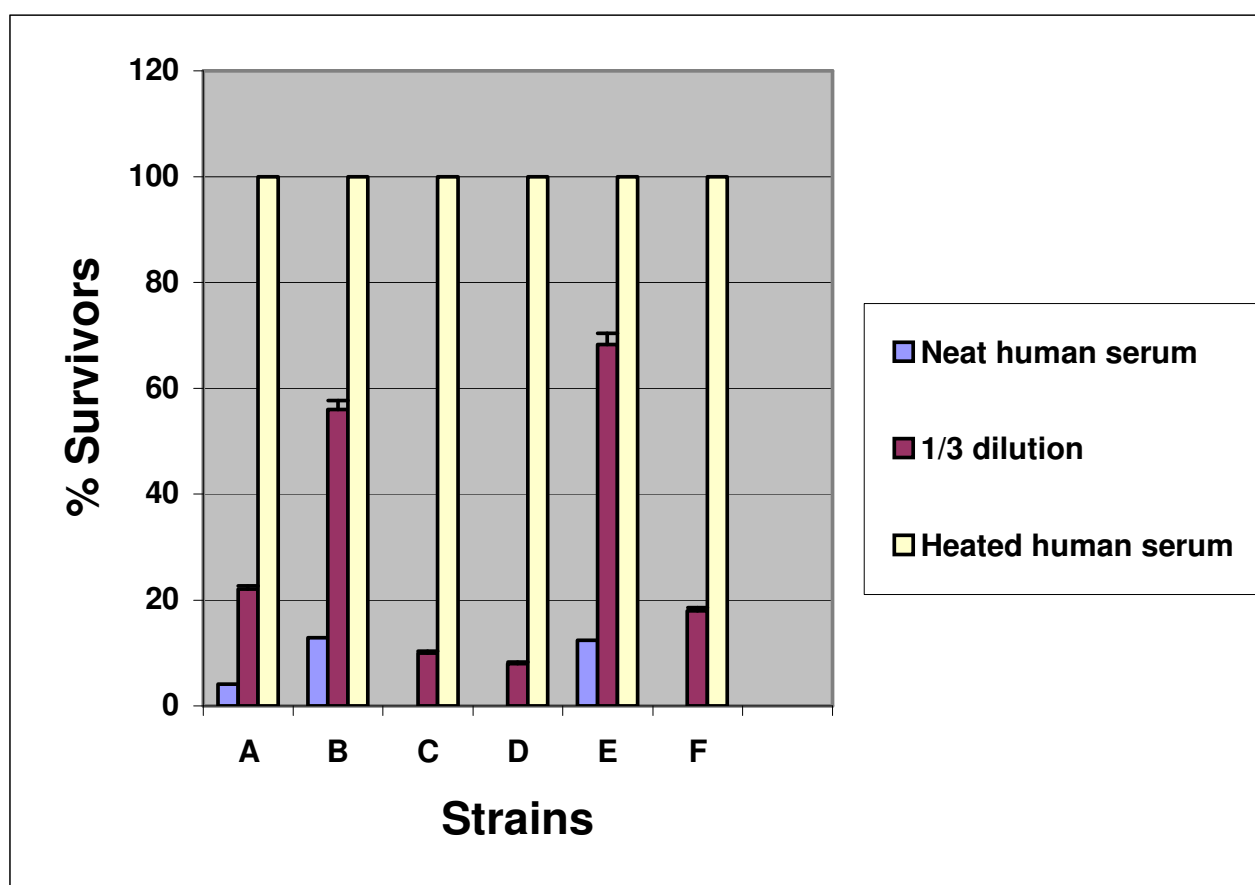


Figure 3.6b Comparison of serum sensitivity of *B. pertussis* strains

Strains were exposed for 45 min to neat or 1/3 dilution of normal human serum (NHS) or NHS that had been heated to 56°C for 30 min. The results are the means of three separate experiments and bars indicate the standard deviations.



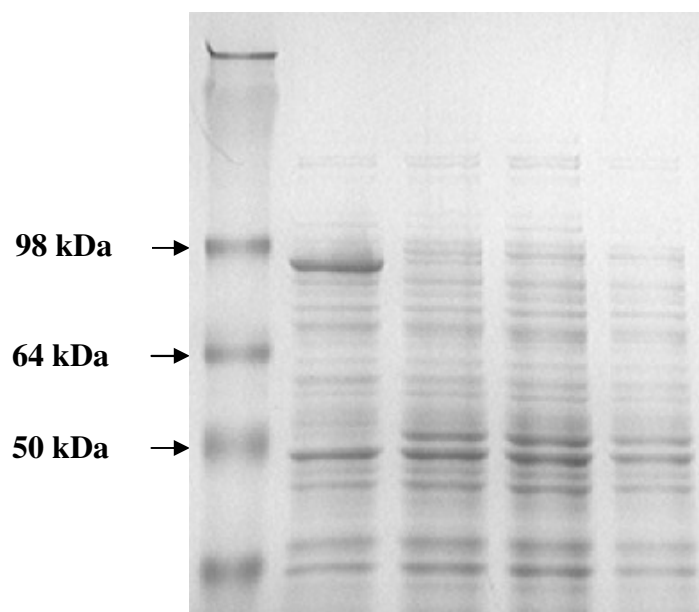
A = BP Tohama (Wild Type)
B = BP338 Weiss
C = BP338 (BrkA⁻) (BP2041 Weiss)
D = BP Tohama II (BapC⁻)
E = BP Taberman (Wild Type)
F = BP Taberman II (BapC⁻)

3.7 Expression and characterisation of recombinant BapC protein from *E. coli* M15 pQE60 (ATG1)

E. coli M15 (pQE60) is a high-level expression strain used for expression of recombinant proteins. For stable propagation of expression constructs encoding potentially toxic proteins, a higher level of *lac* repressor is required which is obtained in *trans* with repressor plasmid pREP4 to express the *lac* repressor at high levels. The *E. coli* M15 (pREP4) strain in induced and non-induced conditions was used as controls.

Fig. 3.7 shows the SDS-PAGE protein profile of whole cells of *E. coli* M15 pQE60 (ATG1) expressing BapC (from the previously predicted first ATG1) in non-induced and induced conditions with IPTG. On this SDS-PAGE 10% polyacrylamide gel, a band at approximately 90 kDa was present in the induced cultured of *E. coli* M15 pQE60 (ATG1) (lane 1) but was absent in the uninduced culture and in the corresponding control preparation from *E. coli* M15 pREP4, without the *bapC* insert. Thus, the band is presumed to be the expected BapC fragment. Previous work had suggested a molecular weight of about 90 kDa for this product (Bokhari, 2002). The predicted molecular weight of this fragment of BapC is 79.5 kDa (see section 3.8) using ProtParam (ExPASy ProtParam) and Biopeptide, which are peptide molecular weight calculator tools. However, the apparent molecular weight of the BapC band according to the calibration of Promega markers (Fig. 3.8) was around 90 kDa. In all cases, protein expression was tightly regulated and was not detectable from uninduced cultures in either Coomassie-blue stained gels or, where performed, immunoblots with anti-His₆ antibodies (section 3.9).

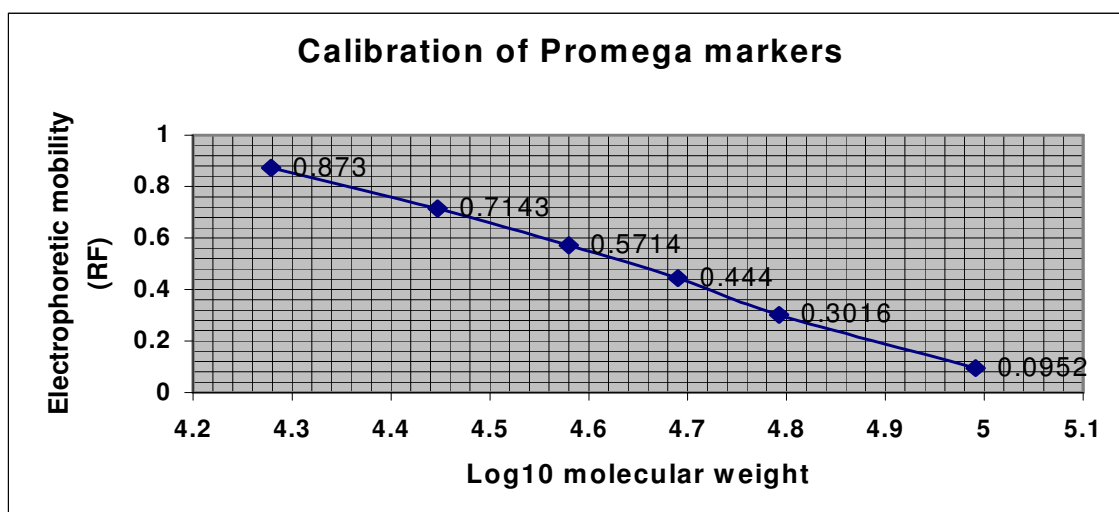
Figure 3.7 Protein profile of *E. coli* strains grown in non-induced and induced conditions with IPTG



- 1 = *E. coli* M15 pQE60 (ATG1)(IPTG⁺)
 2 = *E. coli* M15 pQE60 (ATG1)(IPTG⁻)
 3 = *E. coli* M15 pRep4 (IPTG⁺)
 4 = *E. coli* M15 pRep4 (IPTG⁻)

Figure 3.8 Calibration of Promega protein molecular weight markers (SeeBlue® Plus2 Pre-Stained Standard)

The R_f values for the molecular mass standards were determined by drawing reference lines at the top and bottom of the gel and dividing the distance from the upper reference line to the middle of the protein band (d_1) by the distance from the upper reference line to the lower reference line (d_2). $R_f = d_1/d_2$



3.8 Identification of *bapC* gene and BapC protein in recombinant *E. coli*

To confirm the identification of the cloned *bapC* gene and expression of BapC in *E. coli*, DNA sequencing of the insert and peptide fingerprints of the 90 kDa protein expressed in *E. coli* M15 pQE60 (ATG1) were performed. However, as noted earlier, this plasmid did not contain the full-length *bapC* gene.

The sequence of the cloned *bapC* gene from the purified plasmid of *E. coli* M15 pQE60 (ATG1) carrying the *bapC* gene was obtained using Chromas (versions 2.3 & 1.15) and DNASTar softwares. Then, BLAST analysis of this sequence, using the BLAST2 sequence alignment tools (results not shown), showed the exact nucleotide identity to the *bapC* gene sequence which was in GenBank with accession numbers of AF081494 (Blackburn, 1998) and CAC14167 (Henderson, 2000).

A BLAST search of the peptide sequences obtained by peptide fingerprinting and mass spectrometry of the BapC protein cut from a SDS-PAGE gel (see section 2.11.8) showed a strong homology between the expressed protein and BapC protein (Henderson *et. al.*, 2000) and Bap5 protein (Blackburn, 1998), and strong homology with other autotransporters, such as pertactin and BrkA of *B. pertussis*, putative autotransporters of *B. bronchiseptica* and *B. parapertussis* (Parkhill *et. al.*, 2003) and an autotransporter (pseudogene) of *B. pertussis* Tohama I (Parkhill *et. al.*, 2003). Also, there was an interesting homology (55% identity) between BapC protein and chaperone Hsp70; an auto-regulated heat shock protein of *E. coli*.

Amino acid sequence of BapC by mass spectrometry:

The sequence below is from NCBI BLAST search. Peptides from the expressed BapC protein that matched with this sequence (55% coverage) are shown in bold red, thus confirming the identity of the expressed protein.

Nominal mass (M_r): 79551

MCDTCRDDD G TSPSIR**VQGG VVQGGMGANN VAVVATGSGK VAIENAELLG ASGMYATFGA**
QVDMKGGRIL AHNTNILGSQ GYADGPYGGV VVTEDGQVNL EGAKVSATGL
GAAGLWLLGD KDTSPRASLR NTDVHGEVAA IALGFNGEAN ISGGSLSVED GAVLTTLTPD
 AVEYYYDYAL SMEHLPADAP LTPVRVTLS D GAR**ASGETLI AHGGLPMTL RLSSGVDARG**
 DIVTLPPSAP PDSAEQDAE PEPDAELEPD AAAQSDAKAN ARVMAQVDGG EPVAVPIPAP
 SHPDAPIDVF IDSGAQWRGM TKTVNALR**IE DGTWTVTGSS TVNSLHLQAG KVAYATPAES**
DGEFKHLRVK TLSGSGLFEM NASADLSGD LLVVSDEASG QHKVLVRGAG TEPTGVESLT
 LVELPEGSQT KFTLANR**GGV VDAGAFRYRL TPDNGVWGLE RTSQLSAVAN AALNTGGVGA**
ASSIWYAEGN ALSKRLGELR LDPGAGGFWG RTFAQKQQLD NKAGRRFDQK VYGFELGADH
AIAGQQGRWH VGGLLGYTRA RRSFIDDGAG HTDSAHIGAY AAYVADNGFY FDSLTLRASRF
ENDFTVTATD AVSVRGKYRA NGVGATLEAG KRFTLHDGWF VEPQSEVSFLF HASGGTYRAA
NNLSVKDEGG TSAVLRRLGLA AGRRIDLKGD RVIQPYATLS **WLQEFKGVTT VRTNGYGLRT**
 DLSGGR**AELA LGLAAALGRG HQLYTSYEYA KGNKLTLPWT FHLGYRYTW**

Yellow highlighted segment is the putative cleavage site of BapC protein.

Protein information regarding BapC fragment expressed by *E. coli* M15 pQE60 (ATG1), as obtained from Mascot:

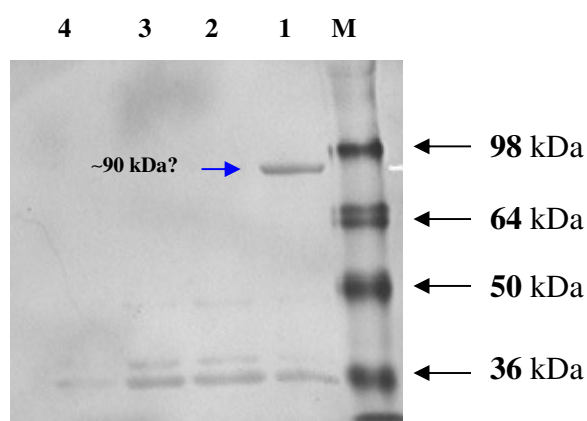
Molecular Weight 79484.46 Daltons
 759 Amino Acids
 64 Strongly Basic (+) Amino Acids (K, R)
 87 Strongly Acidic (-) Amino Acids (D, E)
 274 Hydrophobic Amino Acids (A, I, L, F, W, V)
 179 Polar Amino Acids (N, C, Q, S, T, Y)
 5.132 Isoelectric Point
 -20.408 Charge at pH 7.0

3.9 Characterization of recombinant BapC protein using anti-His-tagged antibody

To detect the BapC protein in *E. coli* M15 pQE60 (ATG1), which is expressing most of the *bapC* gene, anti-His tagged monoclonal antibody was utilised in immunoblotting. It was not certain that the recombinant BapC protein would in fact be His-tagged. In the original reverse primer (Bap-5 CTERM3') used by Blackburn (2000) to produce pQE60 (ATG1) (pQE60 BAPM1, designation of Blackburn) there appears to be a stop codon before the His-tag. Blackburn (2000) was unable to purify the BapC protein by Ni-agarose affinity chromatographs, although Bokhari (2002) did appear to be able to do this. This was further investigated in the present study. Lane 1 (Fig. 3.9) shows the reaction between anti-His-tagged antibody and the recombinant BapC protein fragment, which is expressed

only after induction with IPTG. It appears that the molecular weight of the His-tagged BapC fragment is approximately 90 kDa, as in Fig. 3.7.

Figure 3.9 Immunoblotting with anti-His-tagged antibody of *E. coli* strains grown in induced and non-induced conditions



M = Marker

1 = *E. coli* M15 pQE60 (ATG1)(IPTG⁺)

2 = *E. coli* M15 pQE60 (ATG1)(IPTG⁻)

3 = *E. coli* M15 pREP4 (ATG1)(IPTG⁺)

4 = *E. coli* M15 pREP4 (ATG1)(IPTG⁻)

3.10 Characterization of recombinant BapC protein by immunoblotting

In the immunoblot of the *E. coli* strains grown in induced and non-induced conditions (Fig. 3.10), the unabsorbed anti-BapC serum was clearly reacting strongly with many different components of *E. coli* as well as with the putative BapC fragment (labelled as 79.5 kDa band). To minimize the effect of cross-reacting antibodies in immunoblotting of this recombinant BapC protein expressed by *E. coli*, the BapC serum was absorbed with both *B. pertussis* BP Taberman (BapC⁻) and *E. coli* M15 (pREP4) strains before use.

Figure 3.10 Immunoblotting with unabsorbed anti-BapC serum of *E. coli* strains grown in induced and non-induced conditions

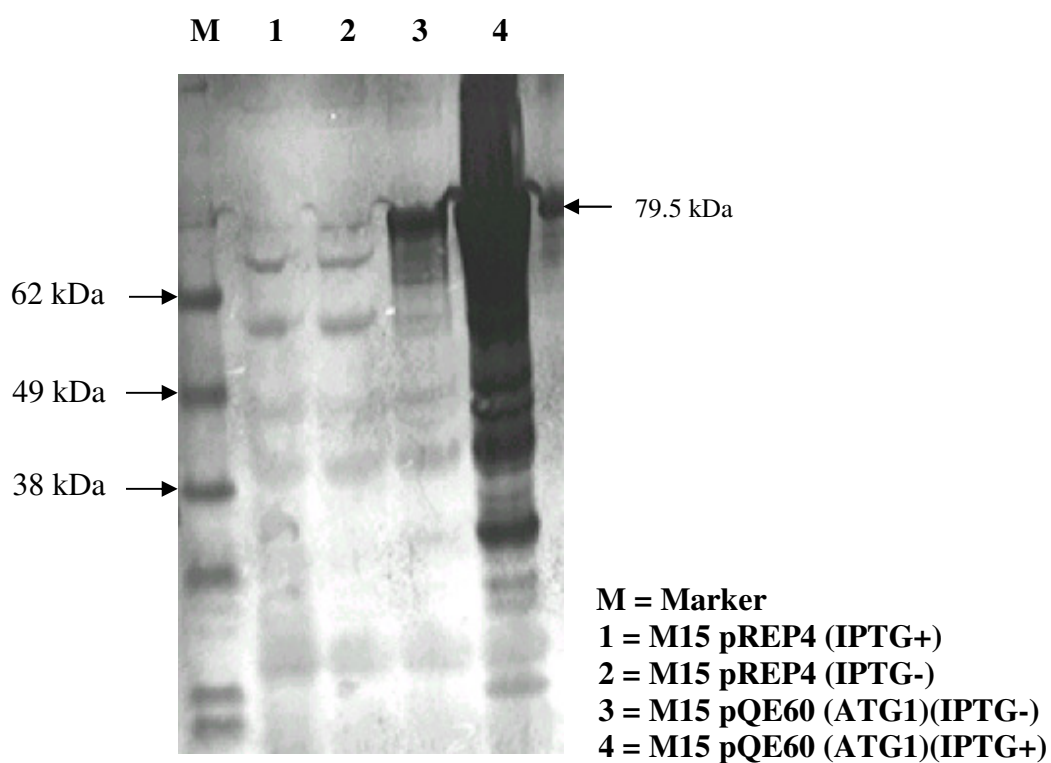
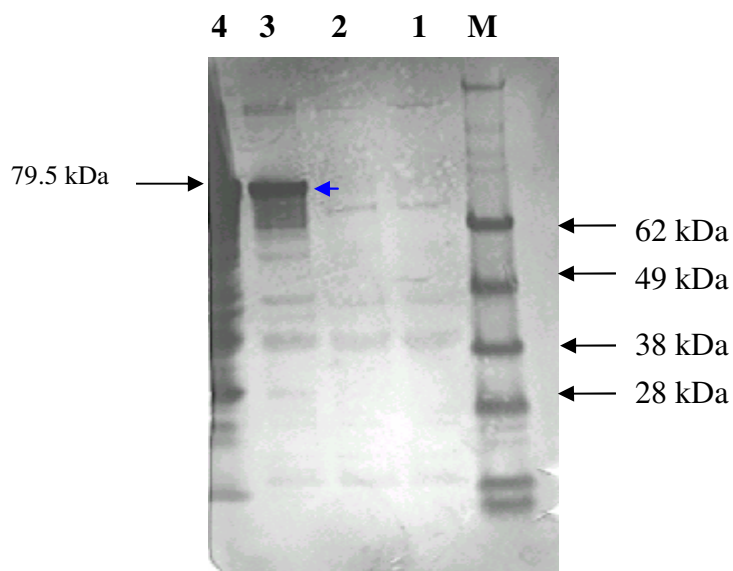


Figure 3.11 Immunoblotting with absorbed anti-BapC serum of *E. coli* strains grown in induced and non-induced conditions.



M = Marker
 1 = *E.coli* M15 pREP4(IPTG+)
 2 = *E. coli* M15 pREP4(IPTG-)
 3 = *E. coli* M15 pQE60(ATG1)(IPTG-)
 4 = *E. coli* M15 pQE60(ATG1)(IPTG+)

Figures 3.10 and 3.11 show the immunoblotting of *E. coli* M15 pQE60 (ATG1) strain with unabsorbed and absorbed sera, respectively, after growth in induced and non-induced conditions with IPTG. Figure 3.10 shows strong cross-reaction of anti-BapC serum with *E. coli* bands, particularly in lane 4, with the induced *E. coli* M15 pQE60 (ATG1) strain. The arrow shows the most likely band corresponding to BapC fragment. Given that the strongest cross-reactions are with pQE60 (ATG1) induced by IPTG, the lower molecular weight bold bands that cross-reacted with anti-BapC serum were presumably breakdown products of the BapC fragment. Figure 3.11 shows the immunoblotting with the absorbed serum, which demonstrates a clear band at approximately 79.5-90 kDa presumed to be BapC fragment and fewer, lower molecular weight cross-reacting bands. This band appeared to be run at somewhere in the region between 79.5 kDa (the predicted size of BapC fragment) and 90 kDa (estimated previously). The estimated size, using the calibrated markers, may not be accurate since any small changes in logarithmic calculations make a huge change in the estimated size of the protein. When compared with figure 3.10, the intensity of the immunoblotting reaction with the main band and lower molecular weight bands has been reduced.

3.11 Localisation of BapC in *E. coli* strains

In previous studies by Blackburn (PhD thesis, 2000) and Bokhari (PhD thesis, 2002) different parts of the *bapC* gene had been cloned into different expression vectors in *E. coli*. When these studies were done, before the publication of the *B. pertussis* genome sequence (Parkhill *et al.*, 2003), the *bapC* gene was assumed to start at position 703 and the corresponding gene fragment was thus named ATG1 (Blackburn, PhD thesis, 2000). A shorter fragment corresponding to the N-terminus of BapC, from positions 778 to 1234 on the *bapC* gene (Appendix II), and cloned into the expression vector, was designated NTS. In the present study, these *E. coli* strains were grown in LB broth and induced with IPTG

(section 2.11.2) and the resulting cell suspensions were tested by slide agglutination with anti-whole pertussis and (unabsorbed) anti-BapC sera.

Table 3.3 shows the agglutination reaction of these different *E. coli* strains expressing most of the *bapC* gene or part of 5' terminus of the gene, with a rabbit polyclonal anti-BapC serum. The polyclonal anti-BapC serum strongly agglutinated the *E. coli* strain expressing BapC [M15 pQE60 (ATG1)] when induced with IPTG and also very weak agglutination when not induced. However, it also cross-reacted to some extent with *E. coli* M15 pREP4 induced or not induced (Table 3.3), which is a parent strain control, and with *E. coli* expressing a part of the N-terminal sequence (NTS) of the *bapC* gene [M15 pQE60 (NTS)], induced or not induced. In fact, none of these strains should express any part of the BapC protein on their surface since the expressed fragment should not be carrying the signal peptide that targets the nascent protein to the inner membrane. However, overexpression of the BapC fragment by *E. coli* M15 pQE60 (ATG1) after induction could perhaps allow some of the protein to reach the cell surface by some unknown means. Moreover, the NTS fragment does not have the β -domain for transport through the outer membrane. Thus, the low level of agglutination of some of these *E. coli* strains could be due to the antigenic cross-reactions already noted and the specificity of the polyclonal anti-BapC serum should be improved for further studies.

In an attempt to further clarify the level of BapC protein expression in *E. coli*, the agglutination procedure was repeated using the rabbit polyclonal anti-BapC serum that had absorbed both with a *B. pertussis* BapC mutant strain and with *E. coli* pRep4 strain. This time, a fine type of agglutination reaction was observed with the *E. coli* strains carrying pQE60 (ATG1) and pRep4 although at lower titres than in Table 3.3. Thus, it seems that the polyclonal anti-BapC antibody was not suitable for detecting the BapC protein specifically in *E. coli*, although it clearly reacts with the protein as shown by immunoblotting experiments (Figs. 3.10 & 3.11). However, when used in agglutination

tests with the various *B. pertussis* strains grown under normal or modulating (Bvg⁻) conditions, the expected results were obtained in most cases (Table 3.2).

Table 3.3 Slide agglutination test with different *E. coli* expression strains in induced and non-induced conditions with IPTG

The agglutination test was performed in 96-well u-shaped microplates using bacterial cells scraped from LB plates and suspended in PBS and the suspension standardized using OD₆₀₀ ~ 0.6.

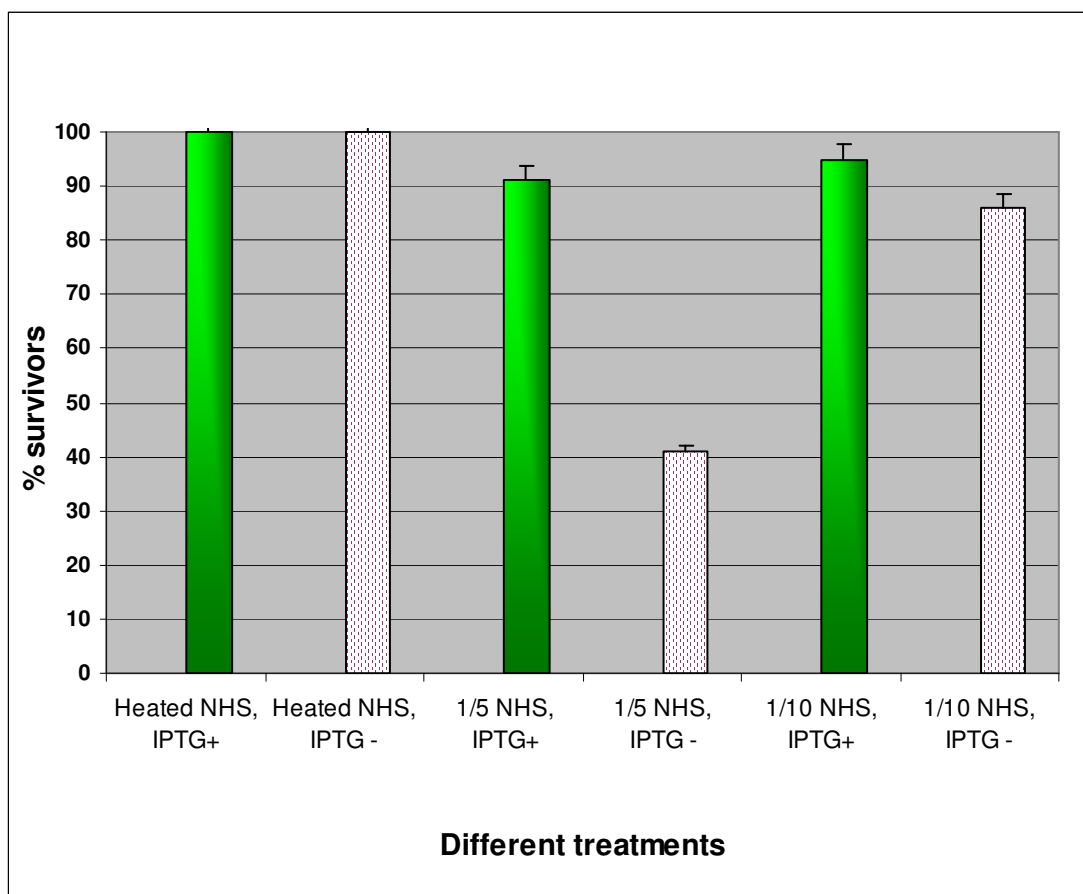
<i>E. coli</i> strains	Agglutination with polyclonal anti-BapC	Self-agglutination control*	Comment
M15 pQE60 (ATG1) (IPTG+)	++++	–	Small clumps of aggregation with clear background
M15 pQE60 (ATG1) (IPTG-)	±	–	
M15 pQE60 (NTS) (IPTG+)	++	–	Fine clumps of aggregation with cloudy background
M15 pQE60 (NTS) (IPTG-)	±	–	
M15 pREP4 (IPTG+)	+	–	Small clumps of aggregation with cloudy background
M15 pREP4 (IPTG-)	±	–	

* Bacteria alone

3.12 Characterisation of BapC as a serum-resistance factor in *E. coli*

This experiment was designed to generate information on the sensitivity to killing by normal human serum of *E. coli* strains containing most of *bapC* gene or part of the *bapC* gene in an expression vector, grown in induced and non-induced conditions. As explained in the previous section, in the light of current knowledge of the *bapC* gene and BapC protein, the *E. coli* strain M15 pQE60 (ATG1) should not have expressed BapC on its surface although the agglutination tests with anti-BapC serum suggested that it did. Expression in *E. coli* M15 pQE60 of most of the whole *bapC* gene (from the previously predicted first ATG1) induced by IPTG did in fact confer significant (P value <0.05) serum resistance to the host strain when compared to the uninduced strain (Fig. 3.12). This difference was especially clear when the strains were exposed to the 1/5 dilution of the normal human serum (NHS), which is approximately the same concentration as in human lungs (Persson, 1991). Thus, even the incomplete BapC protein missing the signal sequence and part of the N-terminal domain, appeared to be reaching the *E. coli* cell surface and conferring serum resistance. However, expression of only part of the N-terminal sequence (NTS) of the BapC protein without the β -domain conferred no significant resistance in the serum killing assay (Fig. 3.13), at either 1/5 dilution of serum or at 1/10 dilution. These figures also show that NHS heated to 56°C for 30 min had no killing effect on the *E. coli* strains or cause any apparent reduction in viable numbers compared to untreated controls.

Figure 3.12 Serum sensitivity of *E. coli* M15 pQE60 (ATG1) with or without IPTG induction

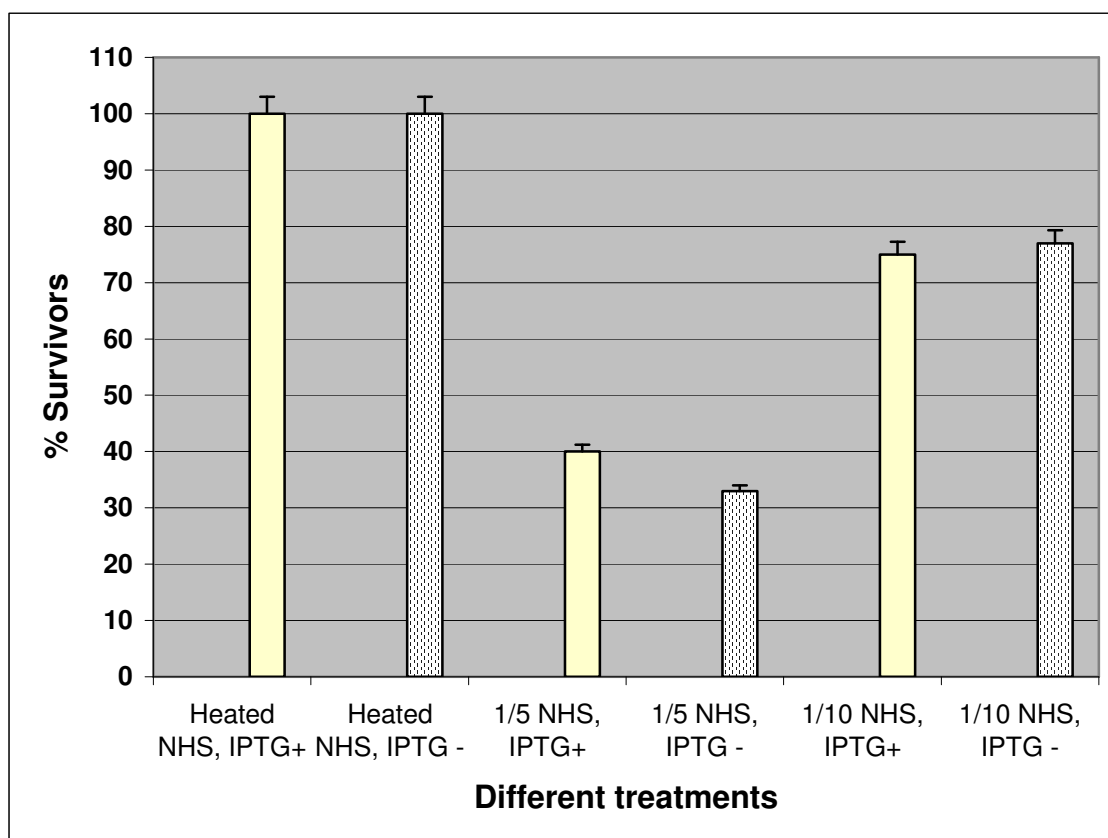


NHS = Normal human serum

IPTG = Isopropylthio-beta-D galactoside

The results are the means and standard deviations of 3 separate experiments. Student's *t*-test was used to compare the strains.

Figure 3.13 Serum sensitivity of *E. coli* M15 pQE60 (NTS) with or without IPTG induction



NHS = Normal human serum

IPTG = Isopropylthio-beta-D galactoside

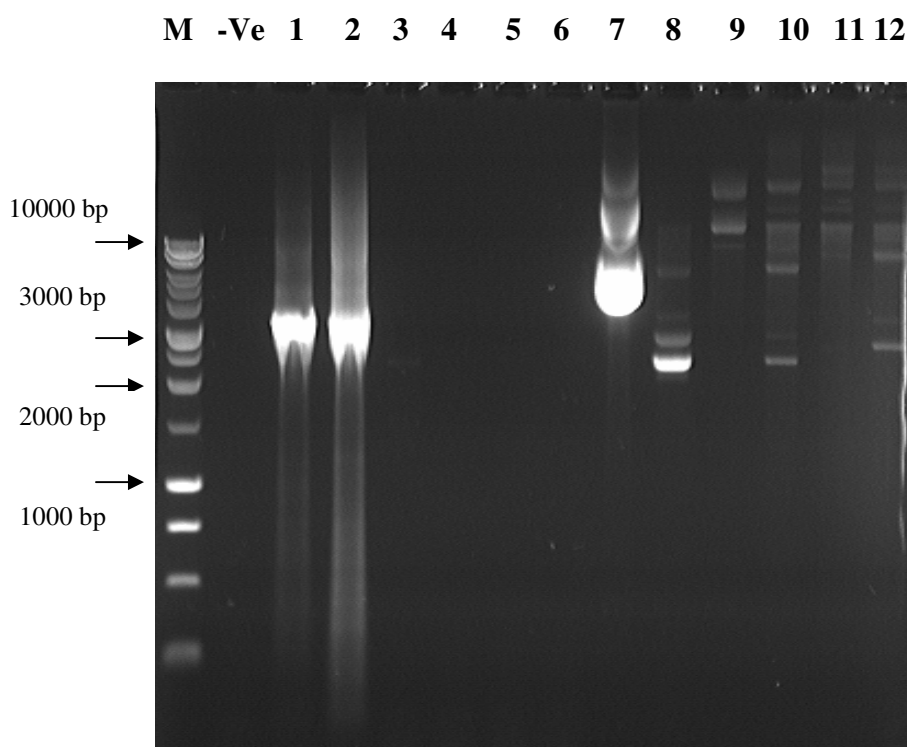
The results are the means and standard deviations of 3 separate experiments. Student's *t*-test was used to compare the strains.

3.13 Identification of cloning and expression vectors

PCR was performed on the different purified plasmids of *E. coli* cloning and expression strains available from previous studies (Table 2.4) to confirm their identities and to show which plasmids were carrying the *bapC* gene disrupted by the *Km* resistance cassette. It was carried out using the *bapC* specific primers, *bapC* forward (BAP5F) and *bapC* reverse (BAP5R) (Table 2.5), which cover most of the full length of *bapC* gene from the ATG at position 728. To make a new *bapC* mutant and also a BrkA, BapC double mutant, it was necessary to confirm the identity of these constructs, which would be useful in the next steps. As figure 3.14 shows, PCR results showed the expected size of 3.5 kbp for the *bapC* gene disrupted by the kanamycin resistance cassette in P-B-5 (Bokhari, 2002) and pCR-Script *bapC::km* (Blackburn, 2000), as suicide and cloning vectors, respectively. These plasmids were used to create a new *bapC* fragment containing Tet^r in place of Km^r. Lanes 3-6 show no band amplified by BAP5F and BAP5R with other (control) plasmid DNAs. Lanes 7-12 show preparations of different plasmids extracted from *E. coli* strains.

Figure 3.14 Agarose (0.8%) gel showing the PCR products and extracted plasmids of some *E. coli* strains with *bapC* specific primers

PCR was performed on plasmid DNA extracted as described in section 2.2.2, using primers *bapC* forward (BAP5F) and *bapC* reverse (BAP5R) which amplify the *bapC* fragment.



M = Marker
-Ve = Negative
1 = P-B-5 (PCR product)
2 = pCR-Script *bapC::km* (PCR product)
3 = *E. coli* JM109 (PCR product)
4 = pSS1129 (PCR product)
5 = *E. coli* M15 pQE60 (NTS) (PCR product)
6 = *E. coli* M15 pREP4 (PCR product)

7 = pCR-Script (*bapC::km*)(plasmid)
8 = *E. coli* JM109 (plasmid)
9 = pSS1129 (plasmid)
10 = pQE60 (ATG1) (plasmid)
11 = pREP4 (plasmid)
12 = pQE60 (NTS) (plasmid)

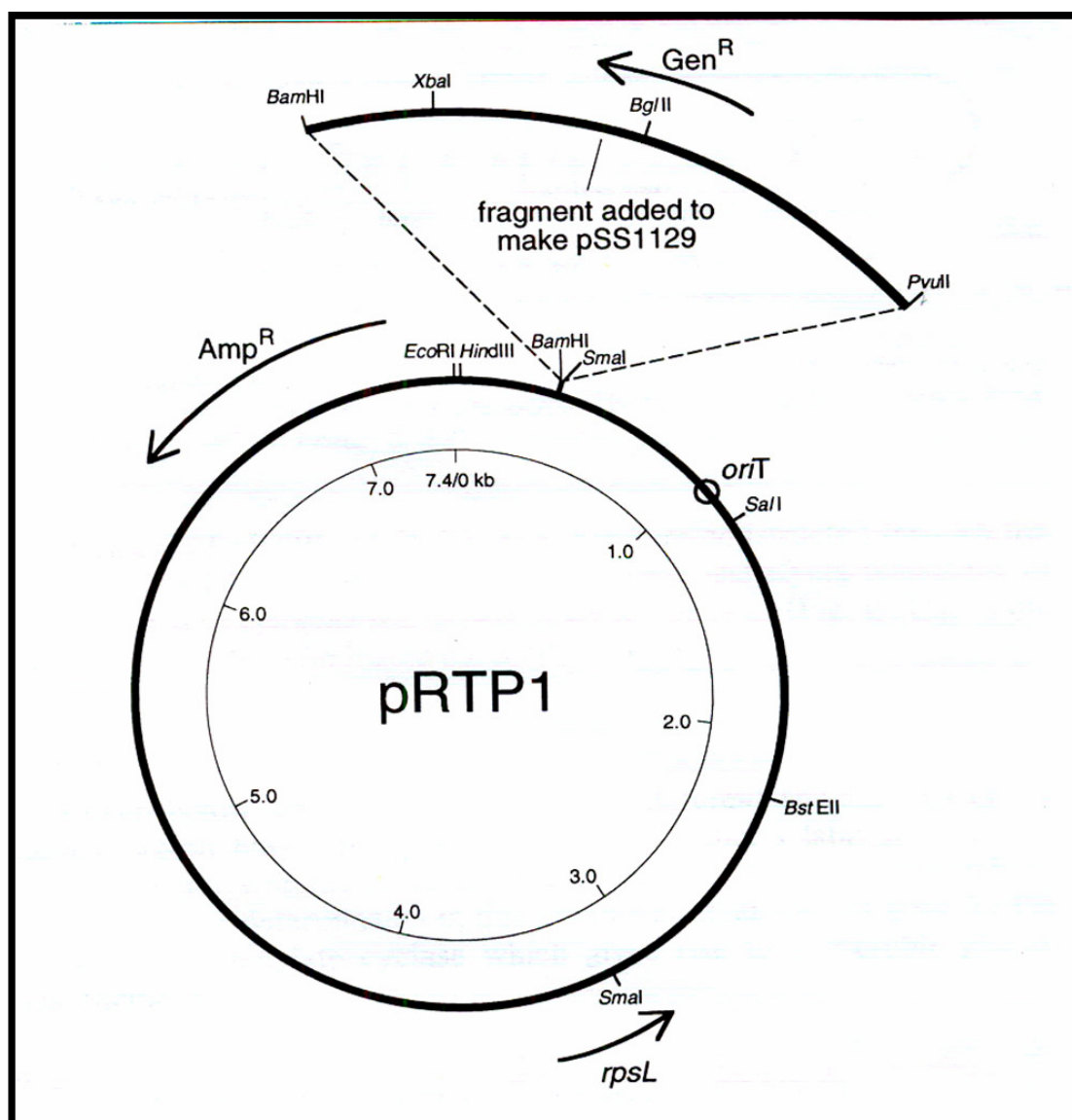
3.14 Generation of *bapC* single and *brkA*, *bapC* double mutants

The suicide vector pSS1129 was utilised to promote allelic exchange of the wild-type *bapC* gene on the chromosome with its *in vitro*-mutated counterpart on the plasmid, by homologous recombination. The construction of pRTP1 has been previously described (Stibitz *et al.*, 1986). This plasmid allows one to introduce a cloned gene into a bacterial recipient by conjugation with an *E. coli* donor and then to select for two successive crossovers between the cloned gene and the recipient chromosome. A 2.4-kb *Bam*HI-*Pvu*II fragment had been added to pRTP1 to make pSS1129 (Fig. 3.15) (Stibitz, 1994). This fragment had been derived from pSK6 (S. Kagan, Ph.D. Thesis, Univ. of Wisconsin, Madison (1981), and the *Pvu*II site and *Sma*I site involved in the cloning were both destroyed.

The important features of this construct are that the transfer to the recipient bacterium can be accomplished because the plasmid contains the origin of transfer from RP4, a promiscuous, IncP plasmid. *Ori*T allows pRTP1 containing cloned *B. pertussis* sequences to be complemented for the ability to transfer via conjugation from *E. coli* into recipient *B. pertussis* strains. When Tra functions are provided *in trans*, pRTP1 will be transferred into the recipient bacterium via conjugation. The pRTP1 also contains a vegetative origin of replication derived from ColE1; an Ap^R gene; the gene for *E. coli* ribosomal protein S12 (*rspL*) which codes RspL protein that is responsible for the streptomycin-sensitive (Sm^S) phenotype in bacterial strains (due to a gene-dosage effect) normally resistant to streptomycin (Sm^R) due to mutation in the chromosomal *rspl* homologue; and the *cos* site of bacteriophage λ .

Figure 3.15 Restriction map of pRTP1 and pSS1129 (Stibitz, 1994)

The 2.4 kbp *Bam*HI-*Pvu*II fragment, which was derived from pSK6, was added to pRTP1 to make pSS1129. Note that the *Pvu*II site and *Sma*I site involved in the cloning were both destroyed.



Once the plasmid DNA has been transferred the vector, it is unable to be maintained in *B. pertussis* due to the ColE1 origin of replication. First crossover will select for those bacteria in which the plasmid has integrated into the chromosome.

If the cloned segment homologous to the chromosome is contained within this plasmid, integration will most likely occur by homologous recombination. If selection for Amp resistance is imposed, only those bacteria in which the incoming plasmid has been integrated into the chromosome will survive.

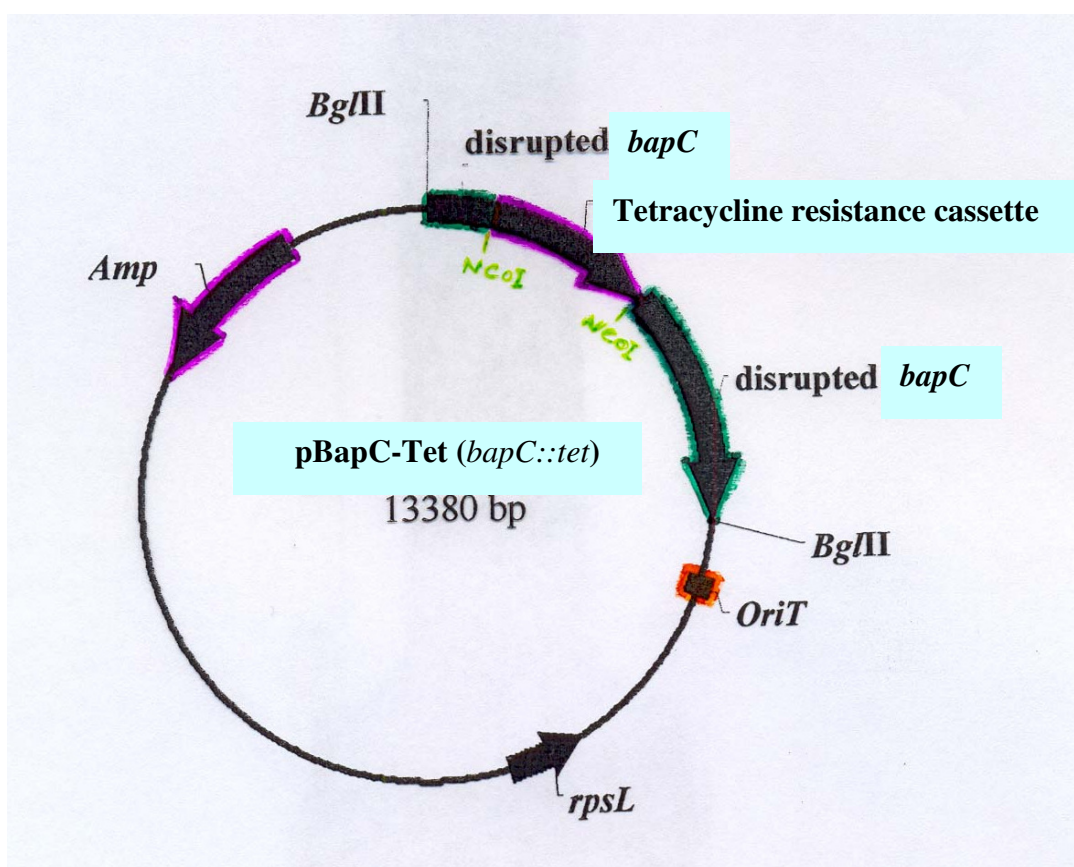
The second crossover is obtained by selecting for the loss of the integrated plasmid. This is accomplished by expression of the wild type *E. coli* *rspL* gene in pRTP1 which confers a streptomycin-sensitive phenotype on strains otherwise resistant due to a mutation in the chromosomal *rpsL* homologue. Thus streptomycin-resistant survivors will be those which will have lost the plasmid copy of *rpsL*. The mechanism by which this loss occurs is via homologous recombination between the direct repeats which flank the vector as a result of its integration. Loss of Amp resistance is indicative of plasmid loss.

3.14.1 Construction of *E. coli* carrying the suicide vector containing *bapC::Tet*

To create a *brkA*, *bapC* double mutant, appropriate antibiotic resistances were required for clonal selection as BP2041 *brkA* mutant was kanamycin and nalidixic acid resistant and also the available *bapC* mutants (BP Taberman II or Tohama II) were kanamycin resistant. A tetracycline resistance cassette was therefore used to replace the kanamycin resistance cassette used previously to disrupt the *bapC* gene at position 1244 (Fig. 3.16). Suicide plasmid pSS1129 and its derivative pBap-5 Kana (P-B-5) (Bokhari, 2002), were extracted by the QIA prep Mini prep purification system (Qiagen), subjected to restriction digestion with *Bgl*III, which cuts either side of the *bapC* fragment inserted (with kanamycin cassette)

Figure 3.16 Map of suicide construct pBapC-Tet [pSS1129 (*bapC::tet*)]

The suicide vector pSS1129 was used to create pBapC-Tet (*bapC::tet*). The *bapC* gene was disrupted by insertion of a tetracycline resistance gene at an *NcoI* site 1244 bp from the predicted translational start site. The vector has a ColE1 origin of replication that is incapable of replication in *B. pertussis* and many other bacterial species. There is an origin of transfer (*oriT*) from the RP4 plasmid. The vector contains the *E. coli rpsL* gene, which encodes streptomycin sensitivity, and an ampicillin resistance gene. The mutated *bapC* gene was cloned into pSS1129 at a *BglIII* site to make the pBapC-Tet (*bapC::tet*) construct.



into the suicide vector. The suicide vector had been employed to create pBap-5 Kana (P-B-5), which is the *bapC* gene disrupted with kanamycin cassette at *NcoI* site, and cloned into pSS1129 at a *BglIII* site (13380 bp) (Kanamycin cassette; 1.3 kbp, *bapC* gene; 2.28 kbp, Suicide vector; 9.8 kbp). Fig. 3.16 shows the map of the suicide construct pBapC-Tet which kanamycin cassette replaced by tetracycline cassette.

Lane 1 in figure 3.17A shows the size of the suicide vector pSS1129 as 9.8 kbp. Lane 2 shows two bands, the *bapC* gene disrupted with kanamycin resistance cassette (3.5 kbp) and suicide vector (9.8 kbp) after digestion of pBap-5 kana (P-B-5) with *BglIII*.

The extracted plasmids were also subjected to restriction digestion with *NcoI*, which cuts either side of the kanamycin resistance cassette inserted into the *bapC* gene (Fig. 3.17B). Lanes 2 shows two bands, the kanamycin resistance cassette (1.3 kbp) and the suicide vector with *bapC* gene (9.8 kbp + 2.28 kbp).

Since the restriction map of the suicide vector was not available, many more restriction digestions were performed to distinguish those enzymes which could cut the suicide vector only in one site without any restriction site in *bapC* (Figures not shown).

To create a new *bapC* mutant and a double mutant of *bapC* and *brkA*; the first step was the construction of the *bapC* gene with a tetracycline resistant cassette. The Tet^R resistance cassette (Tet^R) (Fig. 3.19) was initially amplified from pBR322 (Appendix II) using primers TCF1 and TCR1 (Table 2.5) each of which contained an *NcoI* site (Fig. 3.18A) and the final product was cloned into pGEMT (3 kbp) to create 4.3 Kb pGEMT (Tet). The 1.3 kbp bands in Fig. 3.18A show the amplified tetracycline cassette of plasmid pBR322. The cloned tetracycline cassette in pGEMT vector was sequenced by the Molecular Biology Sequencing Unit of Glasgow University, and BLAST search showed 100%

identity of the insert with the Tet resistance cassette. The 1.3 and 3 kbp in Fig. 3.18B represent the tetracycline resistance cassette and pGEMT plasmid, respectively, after restriction digestion with *Nco*I.

Figure 3.17 Agarose (0.7%) gel showing plasmid pSS1129 and P-B-5 digested with *Bgl*III (A) and *Nco*I (B)

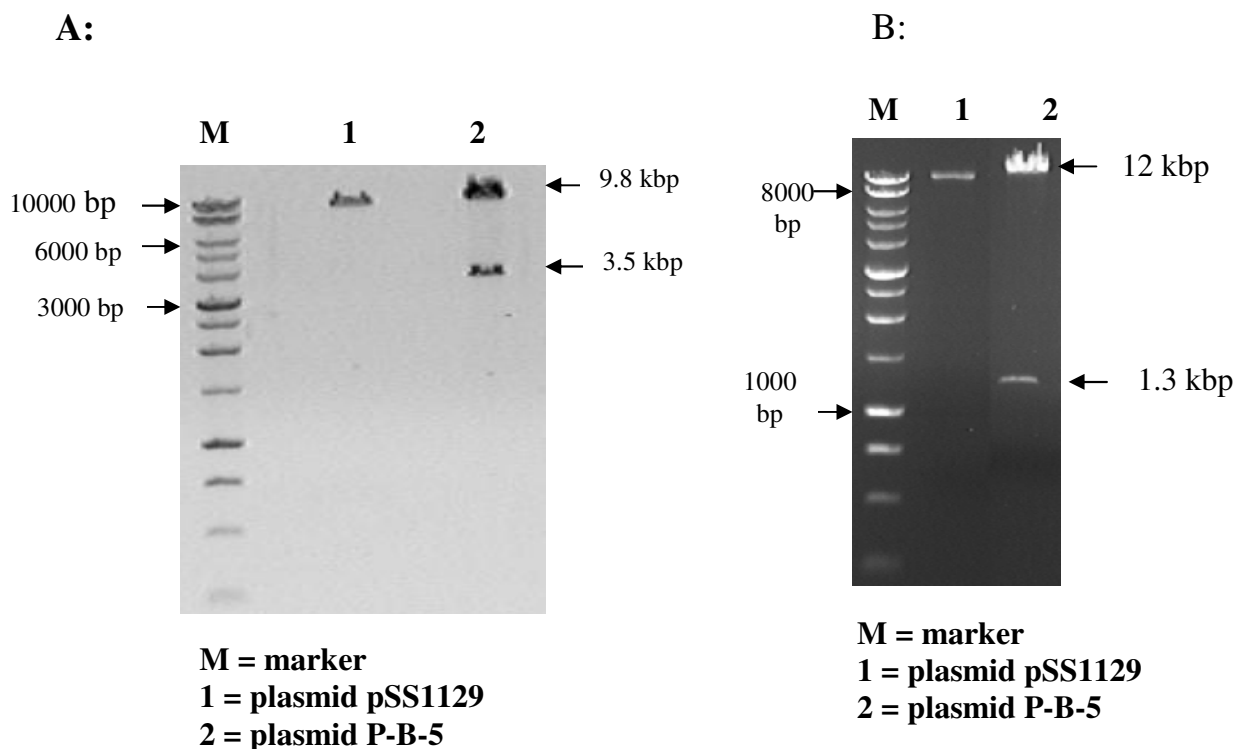


Figure 3.18 Agarose gel (0.7%) showing the PCR product of amplified tetracycline gene of pBR322 (A) and pGEMT(Tet) digested with *Nco*I (B)

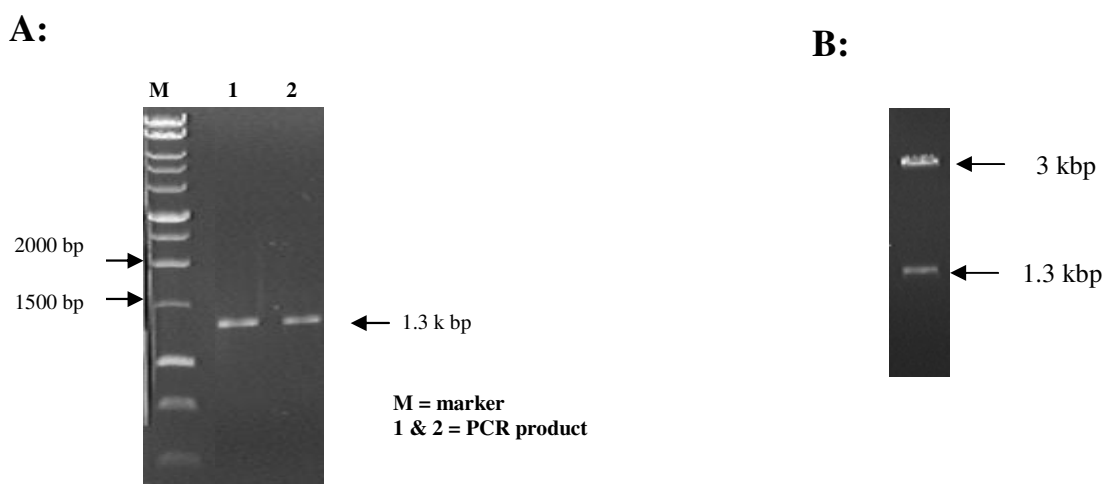
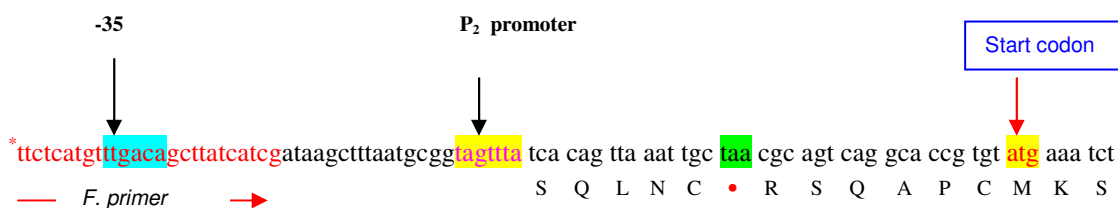
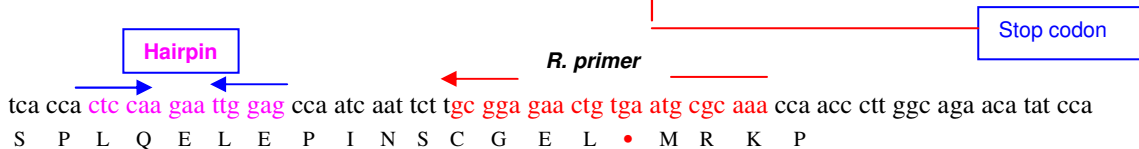


Figure 3.19 Sequence of the tetracycline resistance gene of pBR322
(adapted from www.fermentas.com/techinfo/nucleicacids/mappbr322.htm)



aac aat gcg ctc atc gtc atc ctc ggc acc gtc acc ctg gat gct gta ggc ata ggc ttg gtt atg ccg gta ctg ccg ggc
 N N A L I V I L G T V T L D A V G I G L V M P V L P G
 ctc ttg ccg gat atc gtc cat tcc gac agc atc gcc agt cac tat ggc gtg ctg cta gcg cta tat gcg ttg atg caa ttt cta
 L L R D I V H S D S I A S H Y G V L L A L Y A L M Q F L
 tgc gca ccc gtt ctc gga gca ctg tcc gac cgc ttt ggc cgc cgc cca gtc ctg ctc gct tcg cta ctt gga gcc act atc
 C A P V L G A L S D R F G R R P V L L A S L L G A T I
 gac tac gcg atc atg gcg acc aca ccc gtc ctg tgg atc ctc tac gcc gga cgc atc gtg gcc ggc atc acc ggc gcc
 D Y A I M A T T P V L W I L Y A G R I V A G I T G A
 aca ggt gcg gtt gct ggc gcc tat atc gcc gac atc acc gat ggg gaa gat cgg gct cgc cac ttc ggg ctc atg agc gct
 T G A V A G A Y I A D I T D G E D R A R H F G L M S A
 tgt ttc ggc gtg ggt atg gtg gca ggc ccc gtg gcc ggg gga ctg ttg ggc gcc atc tcc ttg cat gca cca ttc ctt gcg
 C F G V G M V A G P V A G G L L G A I S L H A P F L A
 gcg gcg gtg ctc aac ggc ctc aac cta cta ctg ggc tgc ttc cta atg cag gag tcg cat aag gga gag cgt cga ccg atg
 A A V L N G L N L L L G C F L M Q E S H K G E R R P M
 ccc ttg aga gcc ttc aac cca gtc agc tcc ttc cgg tgg gcg cgg ggc atg act atc gtc gcc gca ctt atg act gtc ttc ttt
 P L R A F N P V S S F R W A R G M T I V A A L M T V F F
 atc atg caa ctc gta gga cag gtg ccg gca gcg ctc tgg gtc att ttc ggc gag gac cgc ttt cgc tgg agc gcg acg atg
 I M Q L V G Q V P A A L W V I F G E D R F R W S A T M
 atc ggc ctg tcg ctt gcg gta ttc gga atc ttg cac gcc ctc gct caa gcc ttc gtc act ggt ccc gcc acc aaa cgt ttc
 I G L S L A V F G I L H A L A Q A F V T G P A T K R F
 ggc gag aag cag gcc att atc gcc ggc atg gcg gcc gac gcg ctg ggc tac gtc ttg ctg gcg ttc gcg acg cga ggc
 G E K Q A I I A G M A A D A L G Y V L L A F A T R G
 tgg atg gcc ttc ccc att atg att ctt ctc gct tcc ggc ggc atc ggg atg ccc gcg ttg cag gcc atg ctg tcc agg cag
 W M A F P I M I L L A S G G I G M P A L Q A M L S R Q
 gta gat gac gac cat cag gga cag ctt caa gga tcg ctc gcg gct ctt acc agc cta act tcg atc act gga ccg ctg atc
 V D D D H Q G Q L Q G S L A A L T S L T S I T G P L I
 gtc acg gcg att tat gcc gcc tcg gcg agc aca tgg aac ggg ttg gca tgg att gta ggc gcc gcc cta tac ctt gtc tgc
 V T A I Y A A S A S T W N G L A W I V G A A L Y L V C
 ctc ccc gcg ttg cgt cgc ggt gca tgg agc cgg gcc acc tcg acc tga atg gaa gcc ggc ggc acc tcg cta acg gat
 L P A L R R G A W S R A T S T M E A G G T S L T D



*Nco*I recognition sequence

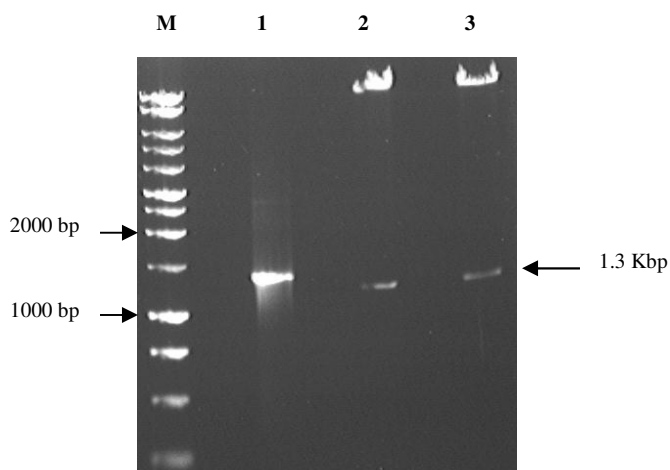
*F. primer: aat c/ca tgg ttc tca tgt ttg aca gct tat cat cg

*R. primer: acg c/ca tgg ttt gcg cat tca cag ttc tcc gc

*Nco*I recognition sequence

During a ligation reaction with PCR-amplified DNA and a plasmid which carries identical termini (e.g. *Nco*I sites), the capacity to circularize and to form tandem oligomers can be avoided by removing the 5'-phosphate groups of the plasmid with alkaline phosphatase and by adjusting the concentrations of the two types of DNA in the ligation reaction to optimize the number of correct ligation products. The 1.3 kbp band (Fig. 3.20) in lane 1 shows the purified PCR product of pBR322 using tetracycline primers (TCF1 and TCR1) then subjected to *Nco*I restriction enzyme digestion. Two bands of 1.3 and 12 Kbp in lanes 2 and 3 are the kanamycin resistance cassette and suicide plasmid, pSS1129, disrupted with *bapC* fragment after digestion of plasmid P-B-5 with *Nco*I restriction enzyme and treatment with alkaline phosphatase, respectively.

Figure 3.20 Agarose gel (0.7%) showing amplified Tet^R gene and products of plasmid P-B-5 after digestion with *Nco*I \pm alkaline phosphatase.



M = marker

1 = Amplified and purified tetracycline gene of pBR322 digested with *Nco*I

2 = Plasmid P-B-5 digested with *Nco*I

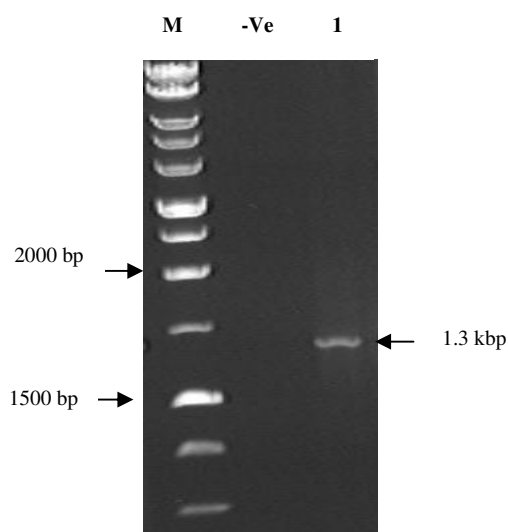
3 = Plasmid P-B-5 digested with *Nco*I and alkaline phosphatase

The Tet^R cassette was purified from pGEMT(Tet) after cutting the plasmid with *Nco*I and inserted at the *Nco*I restriction site of *bapC* (Appendix II.3) already cloned into the suicide vector, leaving enough *bapC* flanking regions for homologous recombination to take place for the construction of the *B. pertussis* *bapC* mutant strains. The ligation of the tetracycline cassette flanking *Nco*I restriction sites into the suicide vector carrying disrupted *bapC* was achieved after many attempts (Fig. 3.21), and the plasmid construct was then transferred by electroporation or heat shock into an *E. coli* host strain, JM109, and into an *E. coli* mobilising strain SM10.λ_{PIR}. Fig. 3.21 shows a 1.3 kbp band by colony PCR using tetracycline primers (TCF1 and TCR1) of a selected colony, from many colonies screened, after successful ligation, electroporation and transformation.

The constructed plasmid was then isolated from the *E. coli* host (JM109) and mobilising (SM10.λ_{PIR}) strains. When the plasmid pBapC-Tet (*bapC::tet*), carrying the *bapC* gene disrupted by the tetracycline resistance cassette, was isolated from *E. coli* JM109 and SM10.λ_{PIR} and digested with *Nco*I, the expected two bands of 12 kbp (pSS1129 + *bapC* fragment) and 1.3 kbp (tetracycline resistance cassette) were obtained (Fig. 3.22 and 3.24A). When the plasmid pBapC-Tet (*bapC::tet*) obtained from these *E. coli* strains was subjected to *Bgl*II restriction enzyme, the expected two bands of 9.8 kbp, the linearised suicide plasmid pSS1129, and 3.5 kbp, the *bapC* fragment disrupted with tetracycline resistance cassette were released (Results not shown).

To further confirm the successful ligation of the mutated *bapC* in the suicide vector, the isolated plasmids were then targeted by PCR reactions using *bapC* primers and Tet primers. Bands of 3.5 kbp (*bapC* fragment + tetracycline resistance cassette) and 1.3 kbp were obtained from the plasmid pBapC-Tet (*bapC::tet*) of *E. coli* strains, JM109 and SM10λ_{PIR}, using *BapC* primers (BAP5F, BAP5R) and Tet primers (TCF1, TCR1), respectively (Figs, 3.23A & B and 3.24B).

Figure 3.21 Agarose gel (0.7%) showing PCR product, using primers TCF1 and TCR1, of a selected colony of *E. coli* containing plasmid with the *bapC* gene disrupted by Tet^R cassette

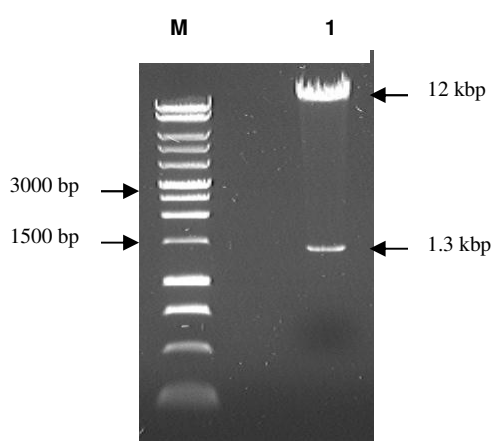


M = marker

-Ve = negative control

1 = PCR product of selected colony of *E. coli* strain after ligation, electroporation and transformation

Figure 3.22 Agarose gel (0.7%) showing plasmid pBapC-Tet (*bapC::tet*) from *E. coli* strain JM109 digested with *Nco*I



M = marker

1 = plasmid pBapC-Tet (*bapC::tet*)

Figure 3.23 Agarose gel (0.7%) showing the amplified *bapC* gene of pBapC-Tet plasmid from *E. coli* strain JM109 (Fig. A), and Nested PCR of amplified *bapC* gene using tetracycline primers (Fig. B).

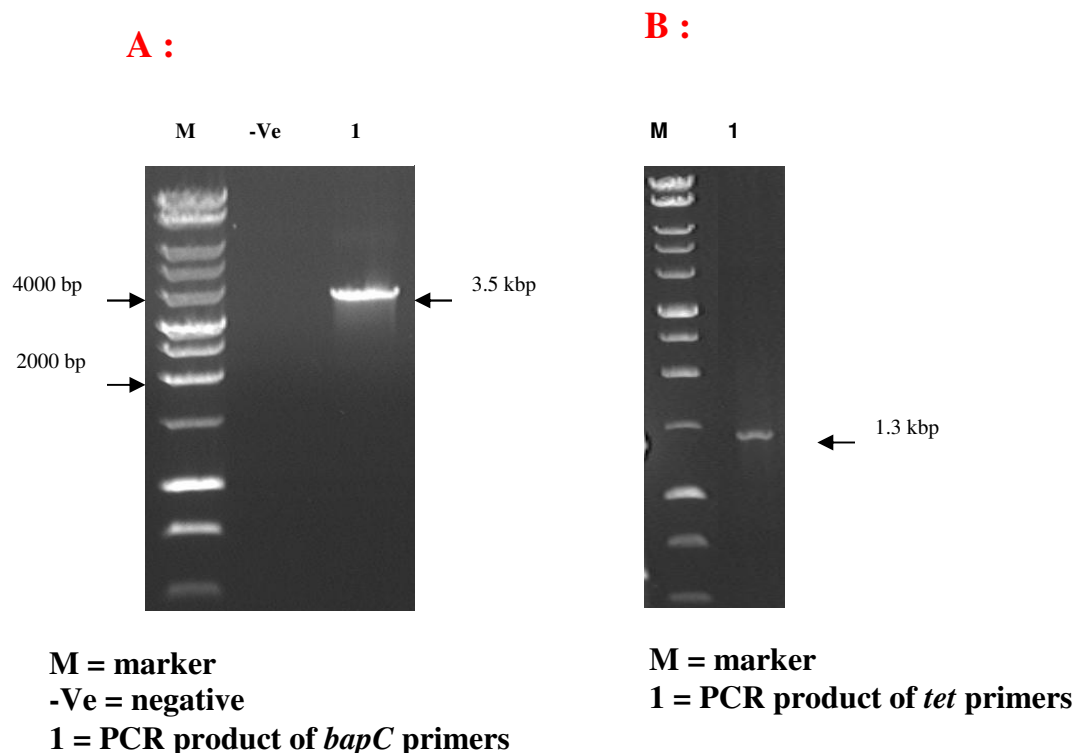
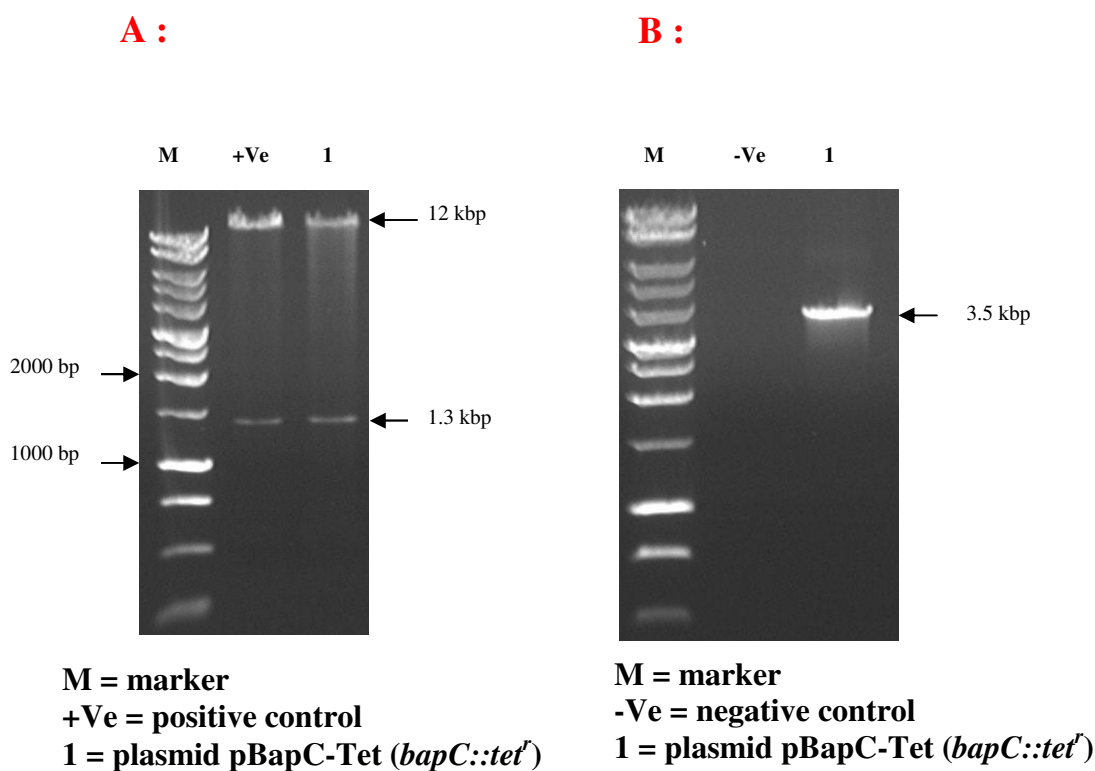


Figure 3.24 Agarose gel (0.7%) showing plasmid pBapC-Tet (*bapC::tet'*) from *E. coli* strain SM10 λ -pir digested with *Nco*I (A) and PCR product of amplified *bapC* gene disrupted by tetracycline resistance cassette (B).

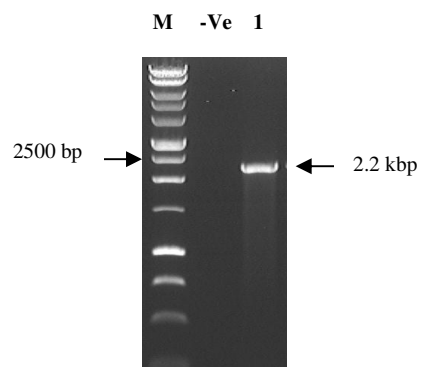


3.14.2 Creation of spontaneous Sm^R derivative of *B. pertussis* BP338 and *brkA* mutant

Spontaneous streptomycin resistant derivatives of BP338 and BP338 ($BrkA^-$) (BP2041) were constructed for use in allelic exchange experiments with the suicide vector pSS1129 carrying *bapC* disrupted with tetracycline cassette (pBapC-Tet) by the procedure described in section 2.7. 10 ml volumes of mid-log phase cultures of BP338 and BP2041 were centrifuged and resuspended in 0.1-0.2 ml of fresh SS medium. Sm^R derivatives of BP338 and BP2041 strains were selected by plating 100- μ l aliquots onto BG agar plates containing 100 μ g of streptomycin per ml. Sm^R colonies of each strain were selected for further studies and were named BP338S and BP2041S. With the exception of Sm^R , BP338S and BP2041S exhibited the same antibiotic susceptibility profiles as BP338 and BP2041, respectively, which strongly suggested that Sm^R was due to an *rpsL* mutation.

The outer-membrane protein profile of BP2041S (Fig. 3.26) was to some extent different from BP2041, but a band (at ~73-kDa) in the wild-type strain BP338, which presumably represents the BrkA protein was absent in both BP2041 and BP2041S. PCR reaction (Fig. 3.25) for the presence of the *bapC* gene using *bapC* primers (BAP5F, BAP5R), showed a 2.2 kbp band, as expected.

Figure 3.25 Agarose gel (0.7%) showing PCR product of streptomycin resistant derivative of *B. pertussis* *brkA* mutant (BP2041S) using *bapC* primers.

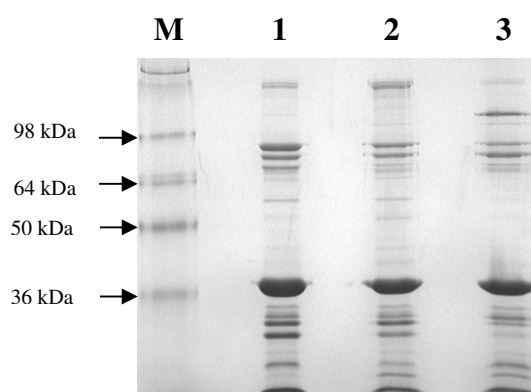


M = marker

-Ve = negative control

1 = PCR product of genomic DNA from BP2041S

Figure 3.26 Outer-membrane protein profiles of *B. pertussis* strains



M = marker

1 = BP338

2 = BP338 (BrkA⁻) (BP2041)

3 = BP2041S

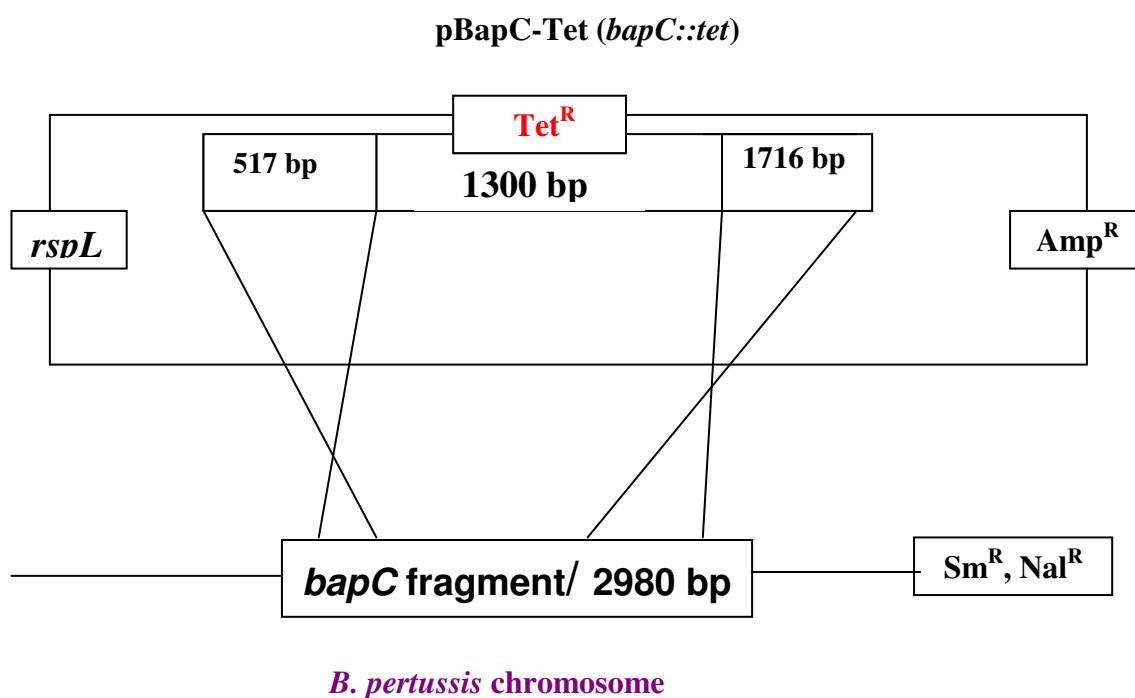
3.14.3 Conjugation to introduce the constructed plasmid into *B. pertussis* strains

Conjugation between the *E. coli* mobilising strain SM10.λ_{PIR} carrying plasmid pBapC-Tet (*bapC::tet^r*) and *B. pertussis* strains (BP338S and BP2041S) was performed according to the procedure given in section 2.7. The exconjugants of BP338S and SM10.λ_{PIR} carrying plasmid pBapC-Tet (*bapC::tet*) were selected by plating onto BG agar containing Nal (40 µg/ml), Tet (2-10 µg/ml) and cephalixin (40 µg/ml) (Fig. 2.1), whereas the exconjugants of BP2041S and SM10.λ_{PIR} carrying plasmid pBapC-Tet (*bapC::tet*) were selected by plating onto Km (40 µg/ml), Nal (40 µg/ml), Tet (2-10 µg/ml) and cephalixin (40 µg/ml). As *B. pertussis* strains are inherently resistant to cephalixin, it was added to the plates to further improve the selection against the *E. coli* donor strain. The survivors of the first selections (single cross-over) were assumed to be *B. pertussis* that contained the plasmid incorporated into the chromosome. The survivors of this step were then restreaked onto BG plates containing Sm (100 µg/ml) to select for loss of the plasmid. The survivors obtained were assumed to be the bacteria that had lost the incorporated plasmid encoding sensitivity to streptomycin due to the second recombination or a double cross-over event, due to homologous recombination between the region of *bapC* present in the plasmid and in the host chromosome.

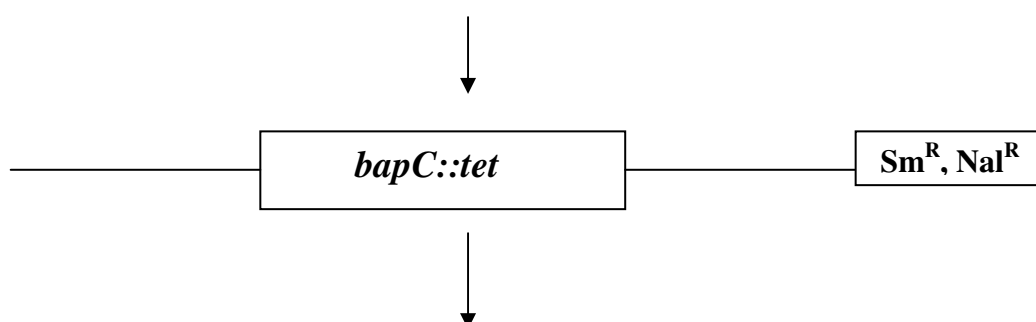
The loss of the integrated plasmid was then confirmed by growing the survivors on BG agar containing ampicillin, a marker in the suicide vector. Those colonies that were sensitive to ampicillin were assumed to be the designed *bapC* mutant and *brkA*, *bapC* double mutant. At the end of these manipulations, two crossovers had occurred between the plasmid-encoded and chromosomal copies of the gene in question. The molecular mechanism of gene replacement in this procedure is summarised in Fig. 3.27.

Figure 3.27 Strategy adopted for replacement of *bapC* gene with *bapC* with inserted tetracycline resistance cassette

1. Single cross-over selection was made by selecting for the plasmid pBapC-Tet (*bapC::tet*)



2. Double cross-over selection was made by selecting against the plasmid using the antibiotic streptomycin and tetracycline

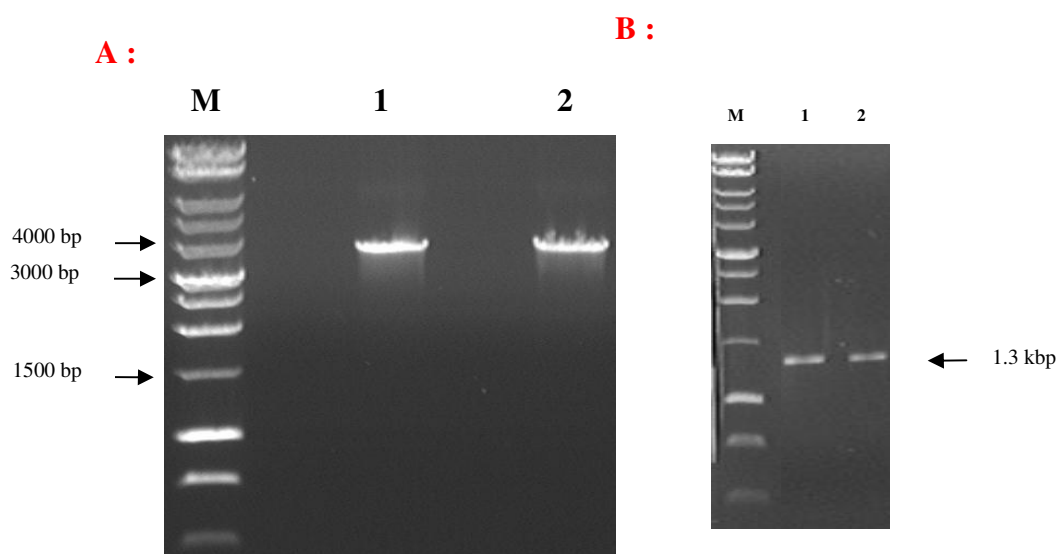


***B. pertussis* chromosome with mutated *bapC* gene**

PCR was carried out on DNA extracted from selected colonies from the final selection to confirm that the wild-type *bapC* gene in the *B. pertussis* strains had been successfully replaced by the mutated *bapC* gene in the plasmid. PCR was performed using the *bapC* specific primers, *bapC* forward and *bapC* reverse (BAP5F, BAP5R) (Table 2.5), which covers most of the length of the *bapC* gene. The PCR results (Fig. 3.28 & 3.29) using genomic DNA extracted (section 2.2.1) from *B. pertussis* 338S and its *bapC* defective mutant, as well as *B. pertussis* BP2041S and its *bapC* mutant, clearly showed that *bapC::tet* of expected size was present in the chromosome of the *B. pertussis* mutant strains. Lanes 1 and 2 in Fig. 3.29 show the PCR products (2.2 kbp) from the parent *B. pertussis* strains BP338S and BP2041S and lanes 1 and 2 in Fig. 3.28A show the PCR products (3.5 kbp) from the *B. pertussis* strains with the mutated *bapC* gene, BP338 (BapC⁻) and BP338 (BrkA⁻, BapC⁻). In the selected clones, there was no evidence of a 2.2 kbp band, representing the native *bapC* gene, from the PCR reactions of the mutated chromosomal DNA. Thus, it was concluded that cross-overs had occurred on each side of the site of Tet^R cassette insertion and the wild type *bapC* allele in the *B. pertussis* chromosome had been completely replaced. If such a double cross-over had not occurred, as shown by the example in Fig. 3.30, there would have been two PCR products obtained from the *B. pertussis* strain in terms of both the wild type allele *bapC* (2.2 kbp) and the mutated allele *bapC::tet* (3.5 kbp).

Further confirmation of the successful constructs was performed using the amplified *bapC* fragment product, after gel extraction, as a template for nested PCR to amplify the tetracycline resistance cassette using Tet primers (TCF1, TCR1). A band of 1.3 kbp, corresponding to the Tet^R cassette, was observed in the *bapC* gene of the BP338 (BapC⁻) and BP338 (BrkA⁻, BapC⁻) (Fig. 3.28B), further confirming the presence of tetracycline resistance cassette in *bapC*.

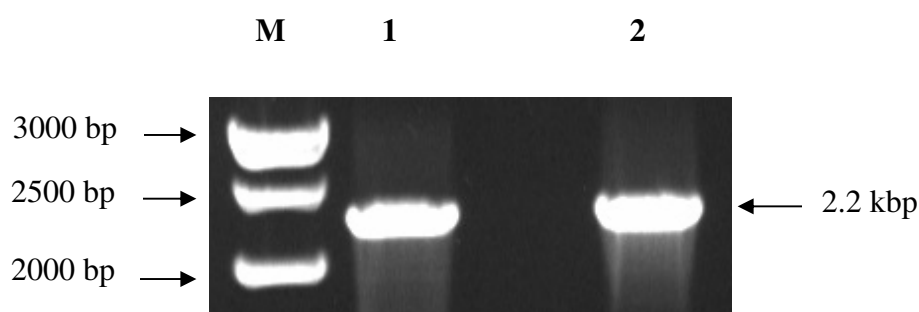
Figure 3.28 Amplified *bapC* gene of BP338 (BapC⁻) and BP338 (BrkA⁻, BapC⁻) mutant strains disrupted by tetracycline cassette (Fig. A) and tetracycline gene inserted in *bapC* gene amplified by nested PCR (Fig. B).



M = marker
 -Ve = negative control
 1 = BP338 (BapC⁻)
 2 = BP338 (BrkA⁻, BapC⁻)

M = marker
 1 = BP338 (BapC⁻)
 2 = BP338 (BrkA⁻, BapC⁻)

Figure 3.29 Amplified *bapC* gene of BP338S and BP2041S strains using *bapC* primers



M = Marker
 1 = BP338S
 2 = BP2041S

Figure 3.30 Amplified *bapC* gene in a selected colony showing a non-double chromosomal cross-over

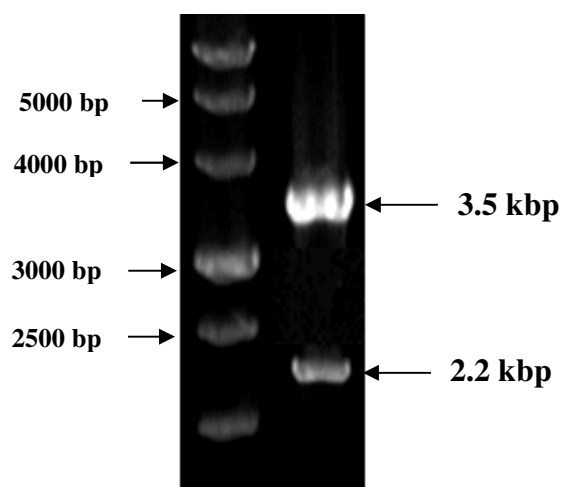
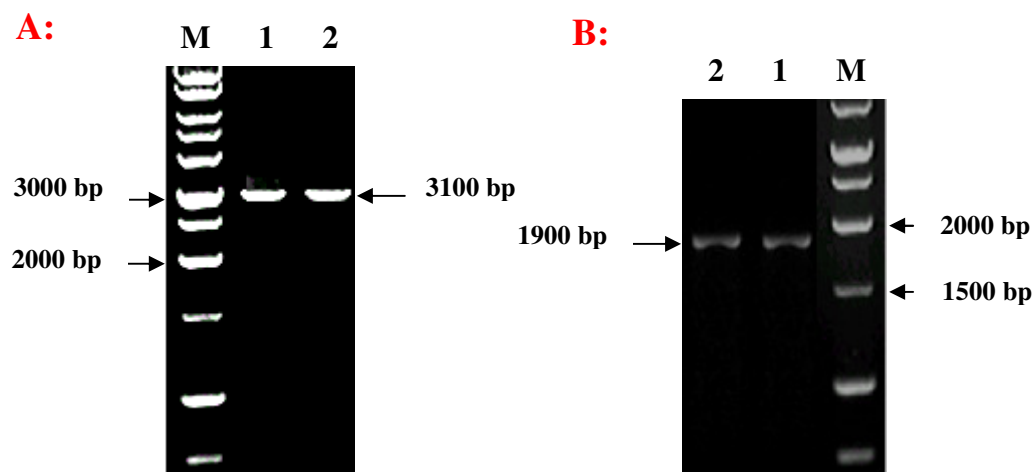


Figure 3.31 5'-terminus (B) and 3'-terminus (A) amplification of the mutated *bapC* gene in constructed mutants using *bapC* forward and Tet reverse primers (B); and Tet forward and *bapC* reverse primers (A), respectively.



M = marker
-Ve = negative control
1 = BP338 (BapC⁻)
2 = BP338 (BrkA⁻, BapC⁻)

Another attempt to confirm the successful constructions was performed by PCR to amplify the 5'-terminus of the mutated *bapC* gene using *bapC* forward primer (BAP5F) and Tet reverse (TCR1) (Fig. 3.31B) and 3'-terminus of mutated *bapC* gene using Tet forward (TCF1) and *bapC* reverse (BAP5R) (Fig. 3.31A), respectively. Amplimers of the expected sizes were obtained.

Further confirmation of the successful construction of the *bapC* mutants was achieved using the Southern blotting procedure. Genomic DNA from the parent and *bapC* mutant strains was digested with restriction enzyme *XhoI* (Fig. 3.32). This enzyme has no restriction sites in *bapC* or in the *Tet^R* locus. The rationale for performing this southern blotting was to probe the digested DNA with a Dig-labelled *bapC*-specific probe, which would confirm the different size of *XhoI* fragments between the parent and mutant strains. Southern blot analysis showed that the specific Dig-labelled *bapC* probe hybridised to an approximately 7.7 kbp fragment in both parent DNA preparations, BP338S and BP2041S, whereas it hybridised to an approximately 9 kbp fragment in both mutant strains, BP338 (BapC⁻) and BP338 (BrkA⁻, BapC⁻). Lanes 3 and 4 in Fig. 3.33 represent the *XhoI* digested genomic DNA from the *B. pertussis* 338S and BP2041S strains, respectively, probed with Dig-labelled *bapC* gene. Lanes 1 and 2 represent the *bapC* mutant strains, BP338 (BapC⁻) and BP338 (BrkA⁻, BapC⁻), respectively, containing the tetracycline resistance cassette insertion into *bapC*. The difference of 1.3 kbp between the parent strains and the *bapC* mutant strains was expected from the insertion of the ~ 1.3 kbp *tet^R* cassette.

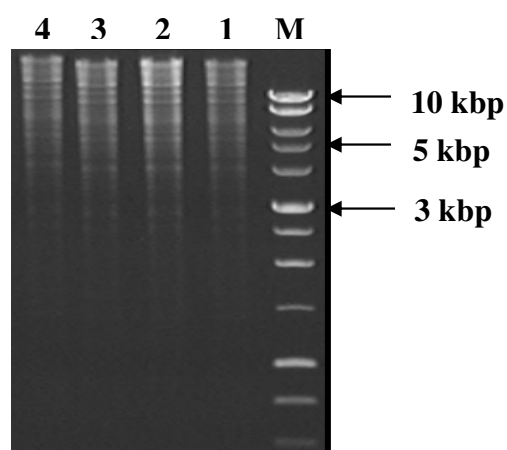
Further confirmation to show the presence of the intact *brkA* gene and the *brkA* gene disrupted by *Tn5* transposon insertion, along with the intact *bapC* and mutated *bapC* genes was performed by multiplex PCR, with strains BP338 and BP338 (BrkA⁻, BapC⁻). Intact *brkA* and *bapC* genes were amplified in BP338 wild type using BrkA and BapC primers (Table 2.5), which amplified 1538 and 2233 bp fragments of the *brkA* and *bapC* genes, respectively (Fig. 3.34). Many unsuccessful attempts were performed using sets of *brkA*

forward primers and *Tn5* reverse primers or *Tn5* forward primers with *brkA* reverse primers (Table 2.5) to show the mutated *brkA* gene. Only non-specific bands were amplified, even using different magnesium chloride concentrations and different PCR parameters as in gradient PCR. The use of *BrkA* primers to show *Tn5* insertion was considered inappropriate as the size of *Tn5* is 5.8 kbp.

Further confirmation of the mutated *BrkA* with the *Tn5* transposon inserted, into position 3232, was therefore done using the Southern blotting procedure. Genomic DNA from the parent, BP2041S, and its *bapC* mutant strain, BP338 (*BrkA*⁻, *BapC*⁻), were digested with restriction enzymes *ClaI*, *EcoRI* and *EcoRV* (Fig. 3.35). These enzymes have unique restriction sites in *brkA* and no site in transposon *Tn5*. The rationale for performing this was that, on probing the digested DNA with a Dig-labelled *brkA* specific probe, a predicted different band size would be detected between the parent and mutant strains. Southern blot analysis showed that the Dig-labelled *brkA* probe hybridised similarly to BP2041S and its *bapC* mutant BP338 (*BrkA*⁻, *BapC*⁻), using *ClaI*, *EcoRI* and *EcoRV* restriction enzymes (Fig. 3.36). The size differences between the parent strains and the *brkA* mutant strains, BP2041S and BP338 (*BrkA*⁻, *BapC*⁻), were as expected from the insertion of the *Tn5* transposon. No differences were seen between BP2041S and BP338 (*BrkA*⁻, *BapC*⁻), as expected.

Figure 3.32 Digestion of genomic DNA of *B. pertussis* strains with *Xho*I enzyme

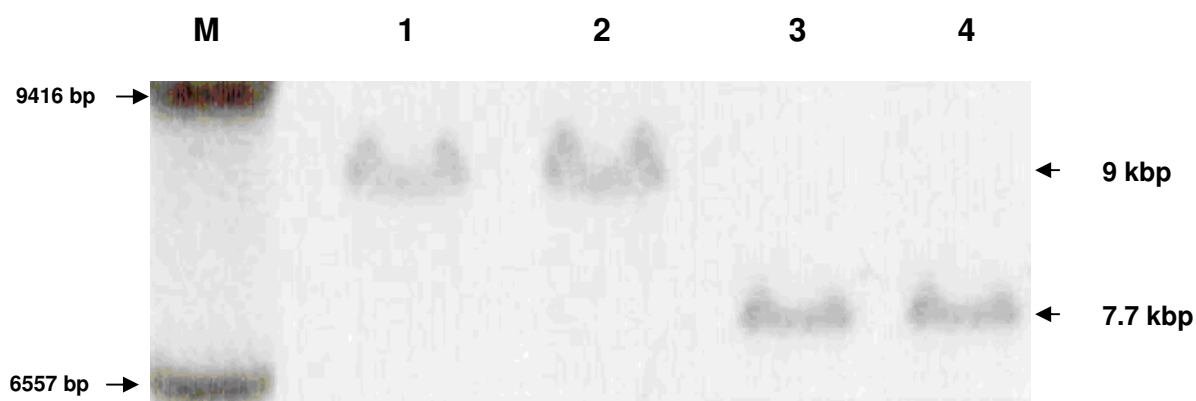
Chromosomal DNA was extracted by the procedure described in section 2.2.1, then subjected to restriction digestion with *Xho*I, which does not cut either the *bapC* gene or the tetracycline resistance cassette, and the fragments were separated on a 0.7% agarose gel.



M = marker
 1 = BP338S
 2 = BP2041S
 3 = BP338 (BapC⁻)
 4 = BP338 (BrkA⁻, BapC⁻)

Figure 3.33 Southern blotting of digested genomic DNA of *B. pertussis* strains with a *bapC*-specific probe

Chromosomal DNA was digested and separated as in Fig. 3.32 and then transferred to a nylon membrane and probed with a Dig-labelled *bapC* gene fragment prepared as described in section 2.10.1. All four samples were run on the same gel and probed on the same membrane. The image is a composite to represent spatially disparate bands.

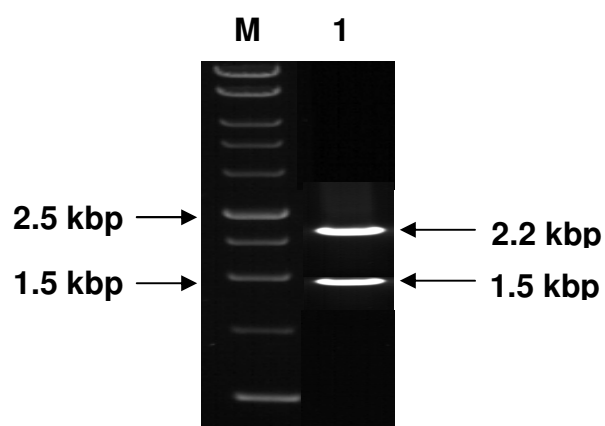


M = marker
 1 = BP338 (BapC⁻)
 2 = BP338 (BrkA⁻, BapC⁻)

3 = BP338S
 4 = BP2041S

Figure 3.34 Agarose gel 0.7% showing amplified wild-type *bapC* and *brkA* genes by multiplex PCR using *bapC* and *brkA* primers.

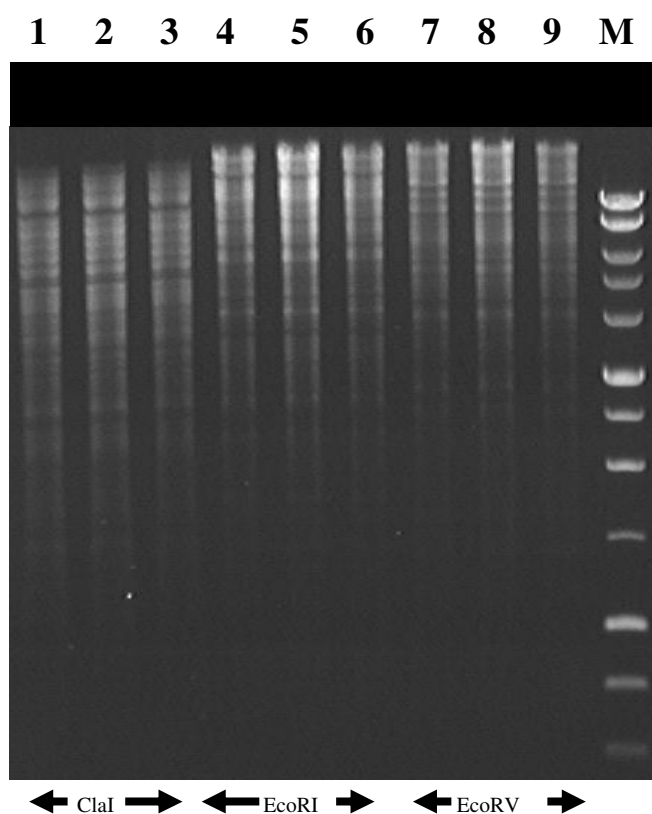
Chromosomal DNA was extracted by the procedure described in section 2.2.1, and then subjected to multiplex PCR with *bapC* and *brkA* primers, which amplify 2.2 kbp and 1.5 kbp fragments, respectively.



M = Marker
1 = BP338 (wild type)

Figure 3.35 DNA genomic digestion of *B. pertussis* strains with *ClaI*, *EcoRI* and *EcoRV* enzymes

Chromosomal DNA was extracted by the procedure described in section 2.2.1, and then subjected to restriction digestion with *ClaI*, *EcoRI* and *EcoRV* enzymes which cut the *brkA* gene at unique sites but do not cut the *Tn5* gene. Fragments were separated on a 0.7% agarose gel.



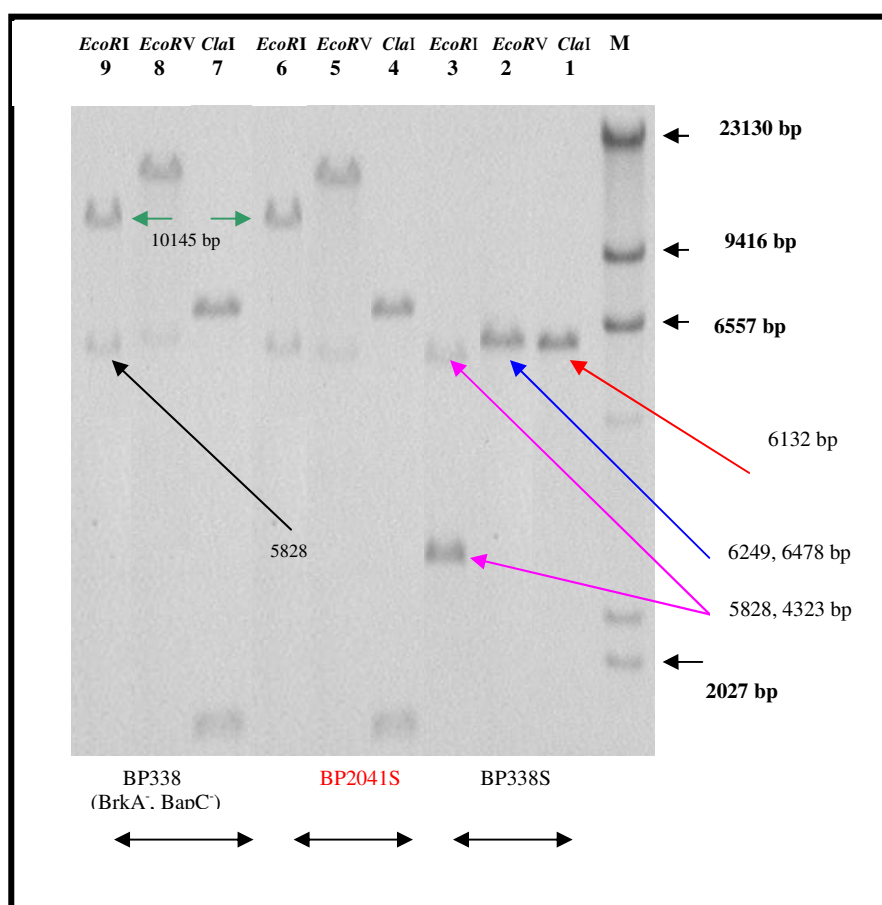
1 & 4 & 7 = BP2041S

2 & 5 & 8 = BP338 (BrkA⁻, BapC⁻)

3 & 6 & 9 = BP338S

Figure 3.36 Southern blotting of digested genomic DNA of *B. pertussis* strains with a *brkA*-specific probe

Chromosomal DNA was digested and separated as in Fig. 3.35 and then transferred to a nylon membrane and probed with a Dig-labelled *brkA* gene fragment prepared as described in section 2.10.1.



3.15 Determining the serum sensitivity of the *bapC* and *brkA*, *bapC* mutants

The rationale for creating the new mutants and for performing serum killing assays was to determine the relative contributions of the BapC and BrkA proteins to the serum resistance phenotype of *B. pertussis*. As in earlier serum killing assays (section 3.6) the assays were performed using human pooled sera (section 2.12.3.1) collected from adult volunteers who had no history of recent pertussis. Heat inactivation, when appropriate, was carried out at 56°C for 30 min. To compare the number of survivors from a PBS control and the heat-inactivated serum as a second control (non-killing control), a preliminary test was performed. There was no significant killing, and no significant difference ($P > 0.05$) between these two controls in a time-course study over 120 min (data not shown). This revealed that any agglutination by antibodies present in the human sera did not significantly affect the bacterial counts.

The standard incubation time for the serum killing assays (usually 45') had been suggested by time-course studies on killing of *B. pertussis* strains with the human serum by Barnes and Weiss (2001) and Bokhari (2002). The survival of the bacteria was calculated relative to a non-killing heat-inactivated serum or PBS (without added serum) control. The percent survival was reported as: $\{\text{the number of bacteria exposed to normal serum}\} / \{\text{the number of bacteria in heat-inactivated serum (or media lacking serum such as PBS)}\} \times 100$. At least three separate experiments were performed for each comparison of wild-type and mutants, and data were analysed by Student's *t*-test. *P* values < 0.05 were assumed to be significant.

Time-course experiments indicated that, after exposure to 1/40 (Fig. 3.37) and 1/80 (Fig. 3.38) dilutions of the pooled human serum (more potent batch than used previously) for 15, 30 and 45 min, *B. pertussis* BP338 strain survived much better compared with its BapC and BrkA mutants or BP Taberman (BapC⁻) mutant. Even after longer exposure to normal

human serum, up to 120 min, in 1/40 and 1/80 human serum dilutions, BP338 was still a better survivor compared to its BrkA or BapC mutants (data not shown). As shown in Figs 3.37 and 3.38 the double mutant was more serum sensitive than either the BrkA or BapC single mutants, but it was not as sensitive as the Bvg⁻ strain (BP347). This finding suggests that other *bvg*-regulated factors might confer or be involved in serum resistance.

The serum killing assays of *B. pertussis* strains in modulating (40 mM magnesium sulphate) and non-modulating conditions (Figs 3.39 & 3.40) also showed that *B. pertussis* BP338 strain appeared to be more resistant to serum killing compared to all other strains at 1/40 serum dilution, as other strains were killed within 15' so there was no discrimination between the serum sensitivity of these strains. Survival of *B. pertussis* BP338 and its BrkA and BapC mutants, grown under modulating conditions, was significantly reduced when exposed to normal human serum compared to that of its counterpart grown under non-modulating conditions.

With 1/80 dilution of normal human serum (NHS), there was greater discrimination between the strains and a clearer picture. BP338 was much more serum resistant than the double mutant, as shown previously (Fig. 3.40), but BP338 and BP338 (BrkA⁻, BapC⁻) strains had similar sensitivities when grown in modulating conditions. Moreover, BP347 (Bvg⁻) showed similar sensitivity whether grown in modulating or non-modulating conditions and was greater than the other strains, even in modulating conditions (Fig. 3.40).

Overall, these data indicated that, in addition to the known role of BrkA in serum resistance, BapC also seems to play a role in conferring resistance on *B. pertussis* against complement killing. A comparison of the double mutant and strains grown in modulating conditions, and with BP347, strongly suggested that other *bvg*- regulated factors may be involved in serum resistance.

Figure 3.37 Comparison of serum killing of *B. pertussis* strains (1/40 human serum)

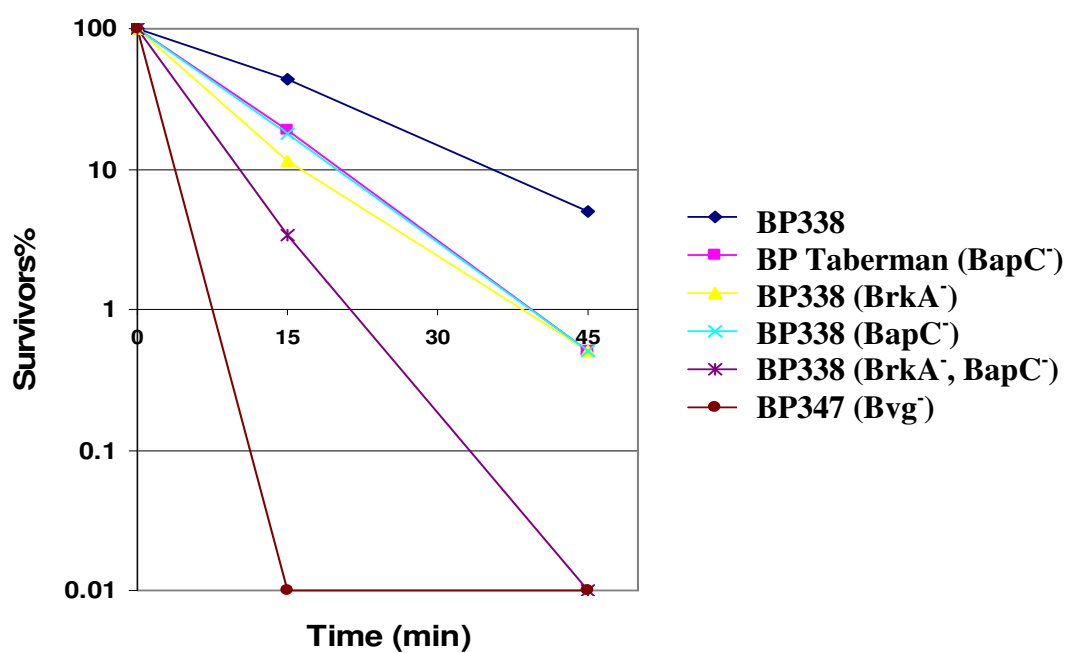


Figure 3.38 Comparison of serum killing of *B. pertussis* strains (1/80 human serum)

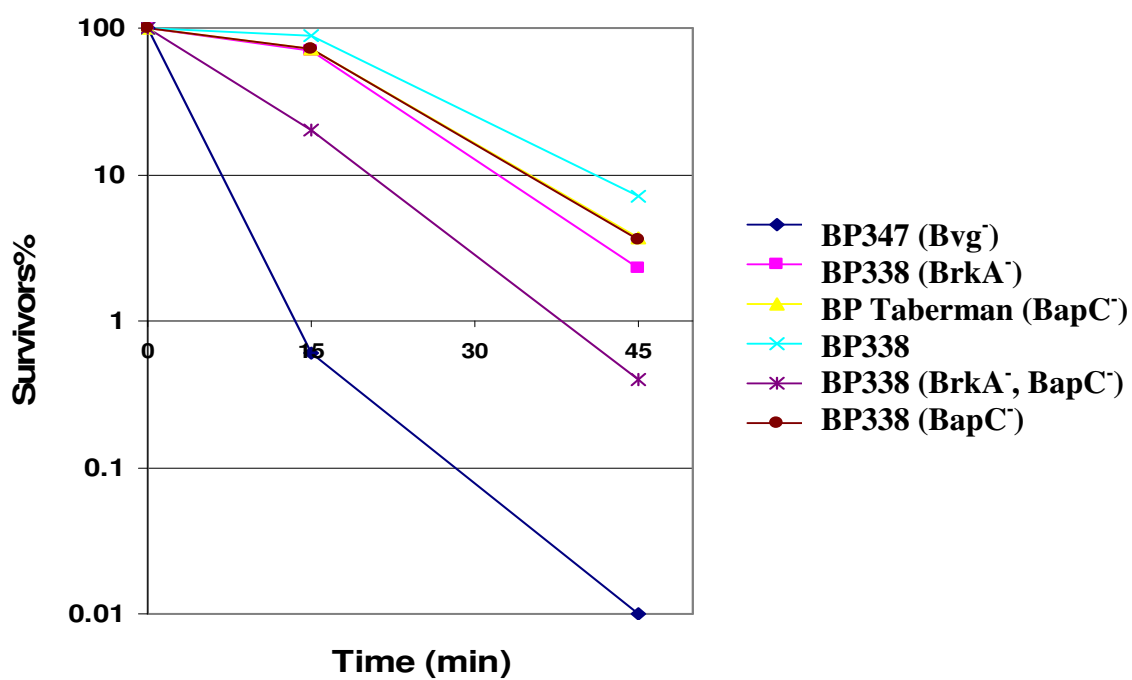


Figure 3.39 Comparison of serum killing of *B. pertussis* strains grown in modulating and non-modulating conditions (1/40 human serum)

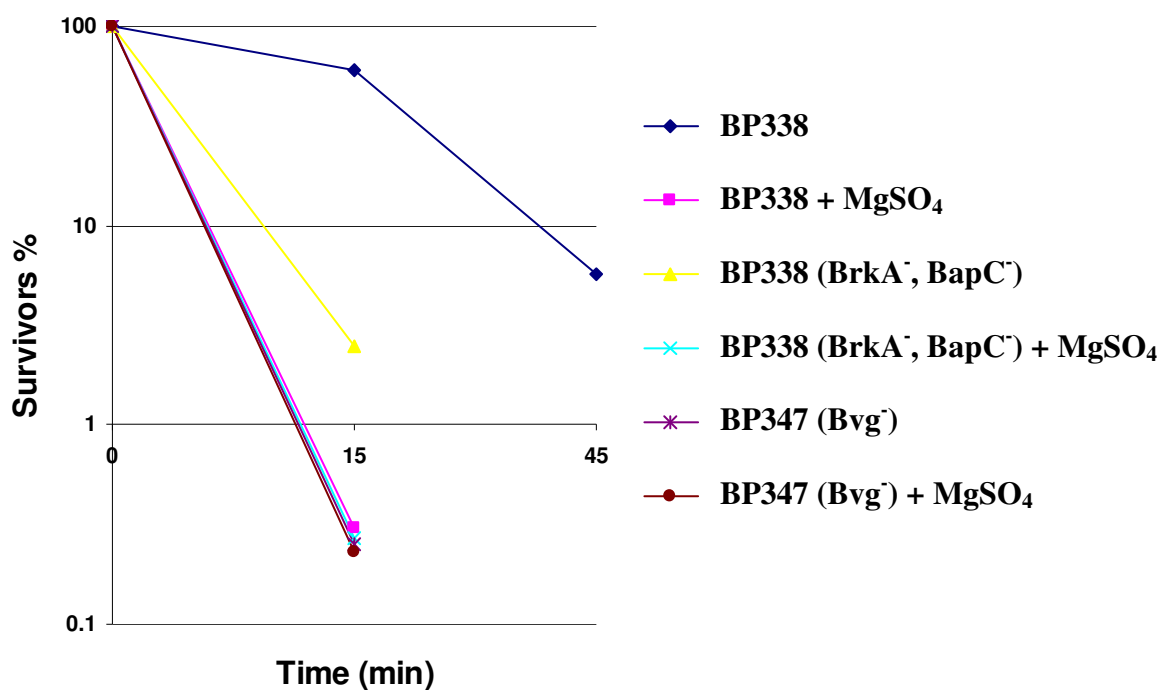
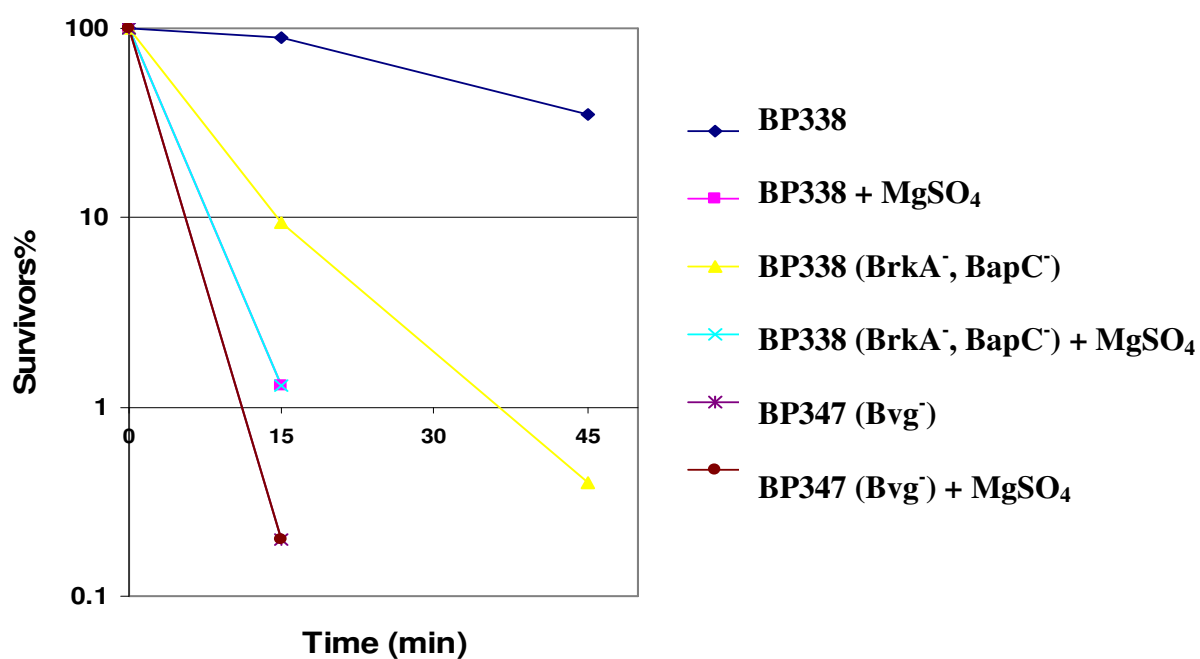


Figure 3.40 Comparison of serum killing of *B. pertussis* strains grown in modulating and non-modulating conditions (1/80 human serum)



3.16 Susceptibility of *B. pertussis* strains to antimicrobial peptides

Antimicrobial peptides are used as effector molecules of the innate immune system. Different groups of antimicrobial peptides are defined, based on structural characteristics. Small cationic peptides with broad-spectrum antimicrobial activity are produced by a wide variety of plants and animals and represent a non-specific arm of the immune systems of these organisms. Antimicrobial peptides are believed to kill bacteria by damaging the cytoplasmic membrane by a mechanism that does not appear to involve interaction of the peptide with discrete protein targets.

In general, antimicrobial peptides are about 12-50 amino acids in size and are predominantly cationic in terms of their net charge. These peptides include two or more positively charged residues provided by arginine, lysine or, in acidic environments, histidine, and a large proportion (generally > 50%) of hydrophobic residues. They exist in four secondary structures, including i) alpha helices (e.g. cecropins) ii) antiparallel beta sheets (e.g. defensins) due to the presence of 2 or more disulphide bonds iii) beta hairpin or loop due to the presence of a single disulphide bond/or cyclization of the peptide chain, and iv) extended. In Gram-negative bacteria, the interaction of these cationic peptides with the negatively-charged lipopolysaccharide-lipid A perturbs the outer membrane. However, the cytoplasmic membrane is the lethal target for antimicrobial peptides, where they can make channels which subsequently results in the disruption of the membrane, which leads to cell death.

It has been shown that the lysis of Gram-negative bacteria by complement is due to the insertion of the C9 component of the membrane attack complex into the cytoplasmic membrane, which is analogous to the mechanism of killing by antimicrobial peptides in that lysis is also dependent on insertion of the peptide into the cytoplasmic membrane.

Fernandez and Weiss (1996) had shown the BrkA, in addition to conferring resistance to serum killing, also conferred resistance to certain antimicrobial peptides such as cecropin P1. Thus, it was decided to examine and compare the susceptibility of the BapC and BrkA mutants to killing by antimicrobial peptides.

The susceptibility of *B. pertussis* strains to various commercially-available antimicrobial peptides was assessed by a modification of the radial diffusion assay described by Lehrer *et al.* (1991). In addition, the concentration of peptide required to kill or inhibit the growth of 50% of the organisms (C-50%) was determined for each peptide in a microtiter plate method and adjusted according to the purity of the peptide stated by the manufacturer. Initially the susceptibility of *B. pertussis* BP338 strain to the different antimicrobial peptides was investigated and varied with the type of peptide. In order of decreasing potency, the C-50% of the peptides was ranked as follow: cecropin P1 > mastoparan > protamine. The susceptibility of the different *B. pertussis* strains, wild type and mutants, to inhibition by antimicrobial peptides was next determined by the radial diffusion assay to find out whether there were any differences in susceptibility.

Exposure of *B. pertussis* strains to the three different antimicrobial peptides resulted in three types of responses. Compared with the wild type strains, the BrkA⁻ strain and the two BapC⁻ strains were more susceptible to inhibition by cecropin P1 and the BP338 (BrkA⁻, BapC⁻) double mutant was even more sensitive (Fig. 3.41). The susceptibility of avirulent strain BP347 (Bvg⁻) was marginally more than that of the BP338 (BrkA⁻, BapC⁻) double mutant, but it was not statistically significant ($p > 0.05$). These findings may suggest that *bvg*-activated factors in addition to BrkA and BapC can mediate some resistance to killing by this peptide. Thus, the sensitivity of *B. pertussis* strains to antimicrobial peptide cecropin P1 was in the order:

Figure 3.41 Susceptibility of *B. pertussis* strains to the antimicrobial peptide Cecropin P1 (0.25 μ M)

The susceptibility of *B. pertussis* strains was compared by using a radial diffusion assay.

Each bar represents the mean and standard deviation of five separate experiments.

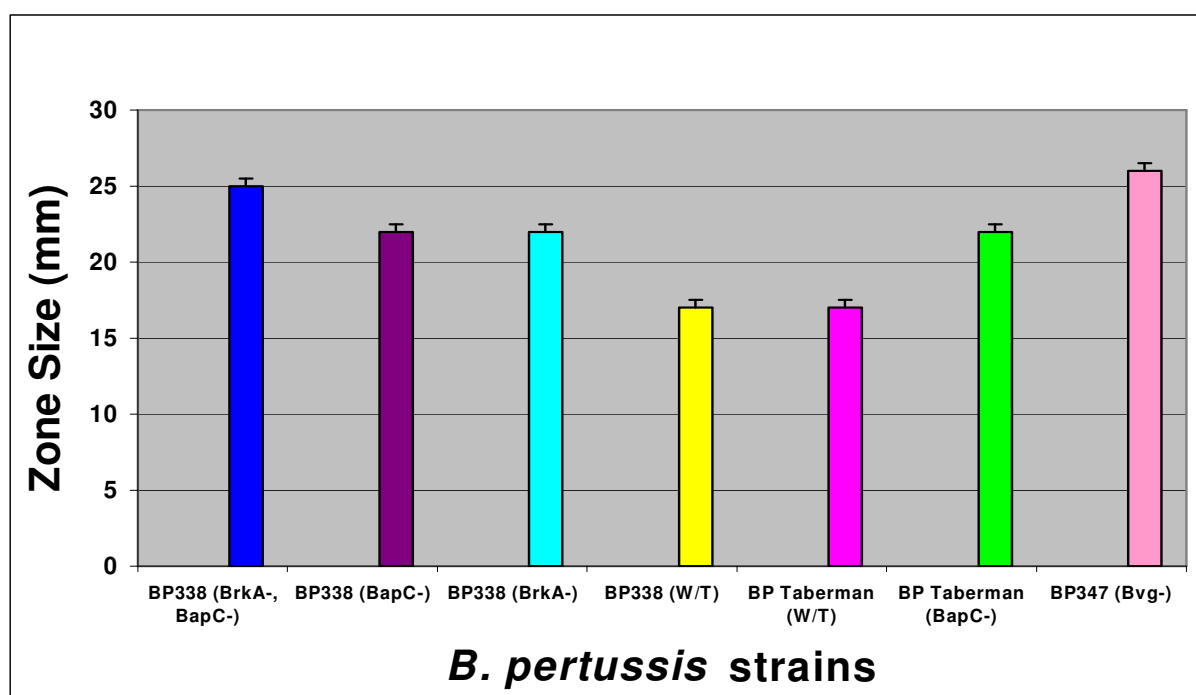


Figure 3.42 Susceptibility of *B. pertussis* strains to the antimicrobial peptide Protamine (2.4 μ M)

The susceptibility of *B. pertussis* strains was compared by using a radial diffusion assay.

Each bar represents the mean and standard deviation of five separate experiments.

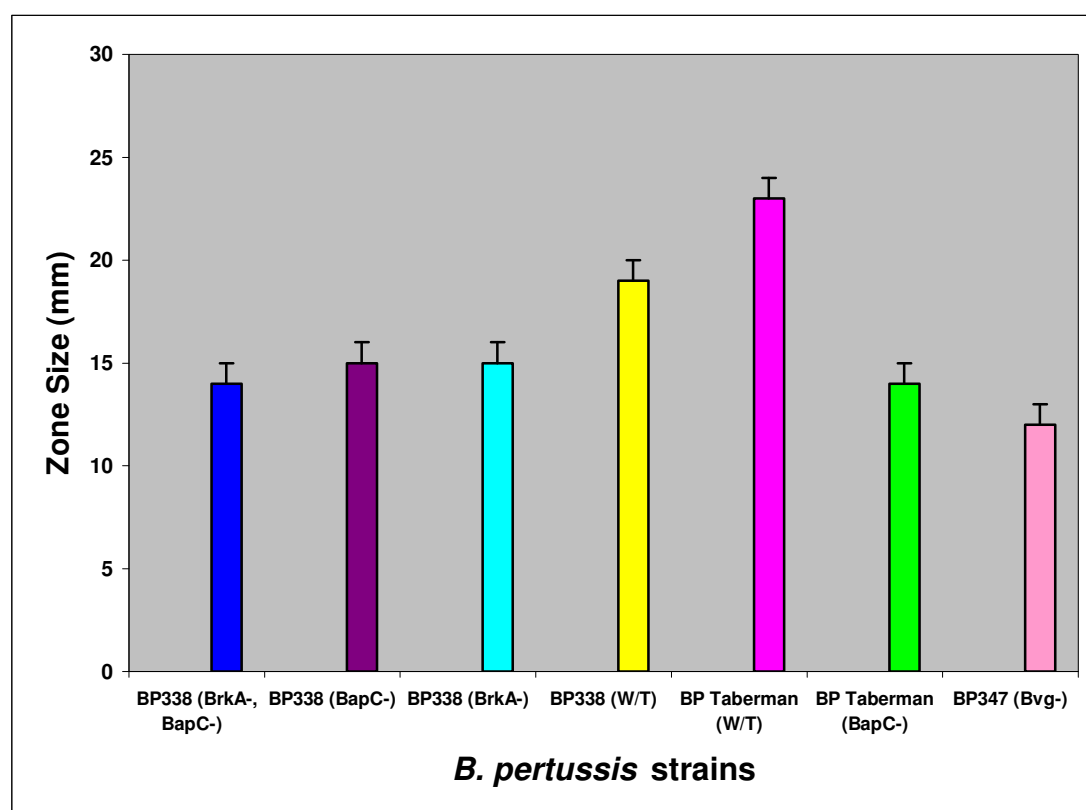
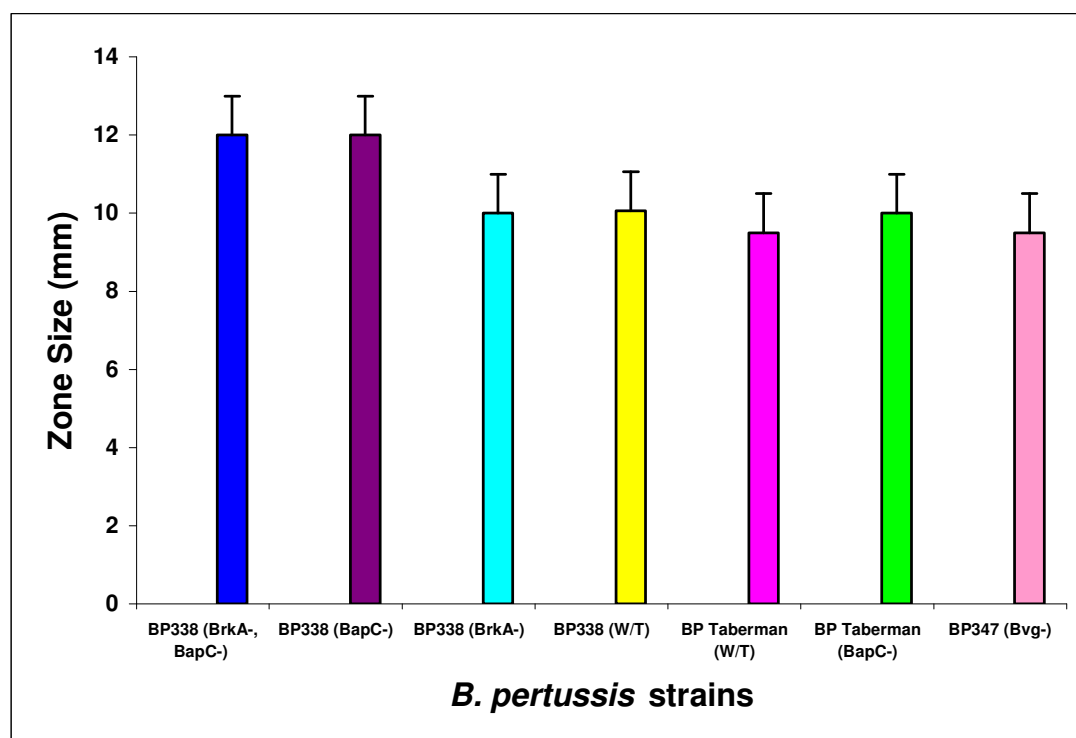


Figure 3.43 Susceptibility of *B. pertussis* strains to the antimicrobial peptide Mastoparan (0.48 μ M)

The susceptibility of *B. pertussis* strains was compared by using a radial diffusion assay.

Each bar represents the mean and standard deviation of five separate experiments.



BP347 (Bvg⁻) > BP338 (BrkA⁻, BapC⁻) > BP338 (BapC⁻) = BP338 (BrkA⁻) > BP338 = BP Taberman

In contrast, the *bvg* mutant strain BP347 was most resistant to inhibition by protamine and the two wild-type strains were most sensitive (Fig. 3.42). The sensitivity of the different *B. pertussis* strains to protamine was in the order:

BP Taberman > BP338 > BP338 (BapC⁻) = BP338 (BrkA⁻) > BP338 (BrkA⁻, BapC⁻) = BP Taberman (BapC⁻) > BP347 (Bvg⁻)

Finally, both BP338 and BP347 were equally susceptible to inhibition by mastoparan (Fig. 3.43). However, the susceptibility of BP338 (BapC⁻) and BP338 (BrkA⁻, BapC⁻) was marginally more than wild-type strains to this antimicrobial peptide, but it was not statistically significant ($p < 0.05$). Overall, the sensitivity of *B. pertussis* strains to the antimicrobial peptide mastoparan was in the order:

BP338 (BrkA⁻, BapC⁻) = BP338 (BapC⁻) > BP338 (BrkA⁻) = BP Taberman (BapC⁻) > BP Taberman = BP347 (Bvg⁻)

It can be concluded that BrkA and BapC can both mediate resistance to inhibition by the antimicrobial peptide cecropin P1 as well as resistance to killing by complement. It is also evident from Fig. 3.41 that, in addition to *brkA* and *bapC*, there could be other *bvg*-activated genes in the wild-type strain that participate in the resistance to killing by cecropin P1, since BP347 is more susceptible than the *brkA*, *bapC* single or double mutants to cecropin P1.

In summary, *B. pertussis* strains exhibited a broad range of susceptibility to different kinds of antimicrobial peptides. For one of these peptides, cecropin P1, expression of BrkA and BapC appeared to confer resistance to inhibition, whereas for protamine, expression of

these factors appeared to confer greater susceptibility. However, it would be of greater interest to examine the susceptibility of the *B. pertussis* to those peptides that are naturally present in the human lungs or trachea, which is the colonisation site by *B. pertussis*, but commercial products were not available at the time of experiment.

3.17 Effect of BapC protein on virulence of *B. pertussis*

There are several autotransporter proteins in *B. pertussis* which are thought to play important roles in the pathogenesis of pertussis. The interaction of *B. pertussis* with ciliated epithelial cells in the respiratory tract is thought to be mediated in part by adhesion proteins such as, pertactin, BrkA and tracheal colonisation factor. It was, therefore, of interest to determine the potential role of BapC autotransporter protein in virulence of *B. pertussis* in a mouse model of infection since BapC revealed some structural homology to the other, better-characterised autotransporters.

To investigate and quantify the degree of virulence attenuation of individual strains, competition assays were performed. In brief, pairs of strains to be compared e.g. wild-type and mutant strains were mixed at a ratio of 1:1, and then inoculated intranasally into randomised groups of five mice. After 7 days, lung counts were done and the output of strain 1 (e.g. mutant strain) CFU compared to the strain 2 (e.g. wild-type strain) CFU was determined by selection on appropriate antibiotics plates. If the ratio of mutant to wild type was the same as in the infecting dose, the mutation had no detectable effect on virulence, but if the wild type out-competed the mutant, the mutation obviously had a negative effect. Comparison of two strains in the same individual mouse helps reduce inter-mouse variation, which may mask small differences in virulence between the two strains.

In preliminary tests, these 1:1 mixtures were simply plated onto BG agar containing 12% horse blood with appropriate antibiotics to show that the selective media could allow selection and enumeration of each strain and that one organism did not affect the growth of

the other *in vitro*. Results are shown in Fig. 3.44 and confirmed that the selection was suitable e.g. that BP338 (BapC⁻) grew equally well on BG agar containing streptomycin as on BG agar and that the count (of BP338 (BapC⁻)) from the 1:1 mixture with the parent strain when plated on BG agar with streptomycin was exactly half that on BG agar. When grown competitively *in vitro* (BG agar with appropriate antibiotics) no defect was observed for the mutant strains during 3 days of culture, showing that the *brkA* and *bapC* mutant strains do not have an inherent growth defect, at least in these growth media (Fig. 3.44).

The randomised groups of 5 mice were inoculated intranasally with approximately 1×10^5 colony forming units/mouse of each strain to be compared, i.e. 2×10^5 CFU/mouse in total (section 2.13.1). The standard mouse virulent strain *B. pertussis* 18-323 was used as a positive control in all experiments. The mice were weighed at regular intervals and sacrificed on day 7. The lungs were then removed aseptically into sterile universal bottles, weighed and then homogenised in CAA solution and plated onto appropriate selective agar, as above. The number of colony forming units (CFU) of each *B. pertussis* strain recovered from the lungs of each mouse were tabulated and competitive indices (CI) calculated. Data were analyzed for statistical significance by using the Student *t*-test or the nonparametric Mann-Whitney test. Statistical significance was defined as $P < 0.05$.

Mouse virulence tests were performed in two separate experiments. In the first experiment, three groups of mice were used and intranasally instilled with either: *B. pertussis* strains BP338 and BP338 (BapC⁻); BP338 and BP338 (BrkA⁻, BapC⁻) and a control group with *B. pertussis* 18-323, a standard mouse virulent strain, alone. In the second experiment, five groups of mice were used and intranasally instilled with either: *B. pertussis* strains BP338 and BP338 (BrkA⁻); BP338 (BapC⁻) and BP338 (BrkA⁻, BapC⁻); BP338 (BapC⁻) and BP347 (Bvg⁻); BP338 (BapC⁻) and BP338 (BrkA⁻) and control group with strain 18-323.

When bacterial counts in the lungs were assessed at 7 days post-infection, there were significant differences between the numbers of bacteria recovered, depending on the mixtures used (Fig. 3.45). The ratio of two strains recovered after infection (corrected for the input ratio) was used to determine whether one strain was out-competed by the other and was therefore attenuated for survival in the host.

The *in vivo* competitive index (CI) was calculated as the reciprocal of (the ratio of strain 1 to strain 2 recovered from the lungs divided by the ratio of strain 1 to strain 2 inoculated into each animal). $CI = 1 / (\text{ratio of strain 1 to strain 2 output CFU} / \text{ratio of strain 1 to strain 2 input CFU})$

Results in Fig. 3.45 show that for example, when BP338 (BapC⁻) strain was compared with BP347 (Bvg⁻) strain, competitive indices of ~0.2 were obtained from all five mice, indicating that far fewer colonies of BP347 were recovered, ~1/5 the number of those of BP338 (BapC⁻). Thus BP347 is much less able to persist in the mouse lung than the BapC mutant ($P < 0.05$). In brief, the *in vivo* analysis revealed that the Bvg⁻ strain (BP347) was the least virulent of the strains. The data also indicated that BP338 was far better in colonising the mouse lung than its *bapC*, *brkA* single and *brkA*, *bapC* double mutants ($P < 0.05$). The data also showed however that the double mutant BP338 (BrkA⁻, BapC⁻) was less virulent than either BapC or BrkA single mutants alone, as revealed by a lower survival rate of the double mutant strain from infected mice compared to the single mutants ($P < 0.05$), although it was not so low in virulence as BP347 (Bvg⁻). The recovered lung count for *B. pertussis* 18-323, the standard mouse-virulent *B. pertussis* strain used for vaccine tests, was significantly ($P < 0.05$) higher than for BP338 (data not shown). Overall, the mouse virulence of the test strains was in the order as follows:

BP338 (W/T) > BP338 (BrkA⁻) > BP338 (BapC⁻) > BP338 (BrkA⁻, BapC⁻) double mutant > BP347 (Bvg⁻)

Figure 3.44 Effect of growth of two strains input mixture on BG agar

B. pertussis strains were grown on BG agar containing appropriate antibiotics for 3 days and then lawn culture made for further 24h. The resultant was suspended and adjusted by serial dilution to 1×10^3 CFU/ml in 1% CAA, and 0.1 ml of two strains to be compared, were plated in duplicated on BG agar plates. Colonies were counted after 3-4 days. The data presented are the means and standard deviations.

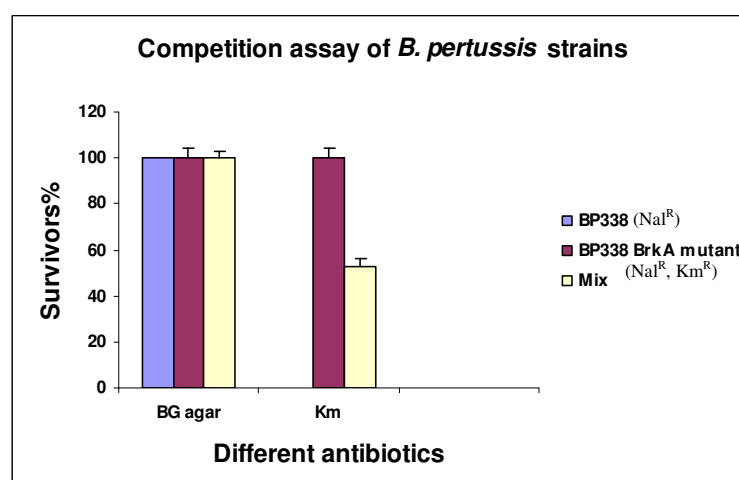
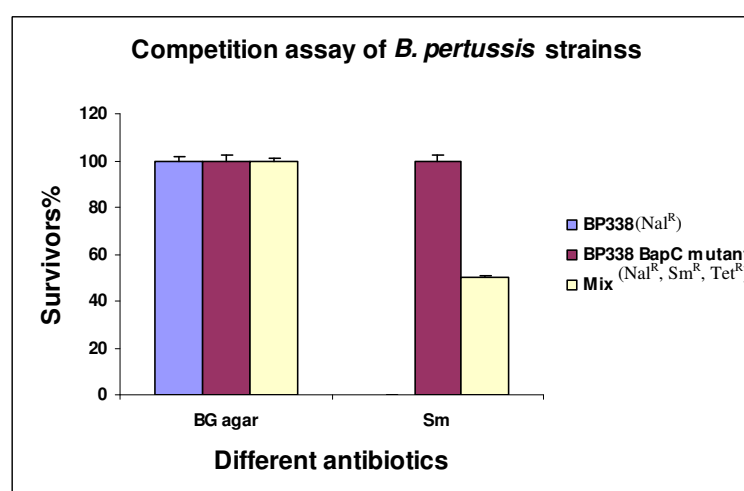
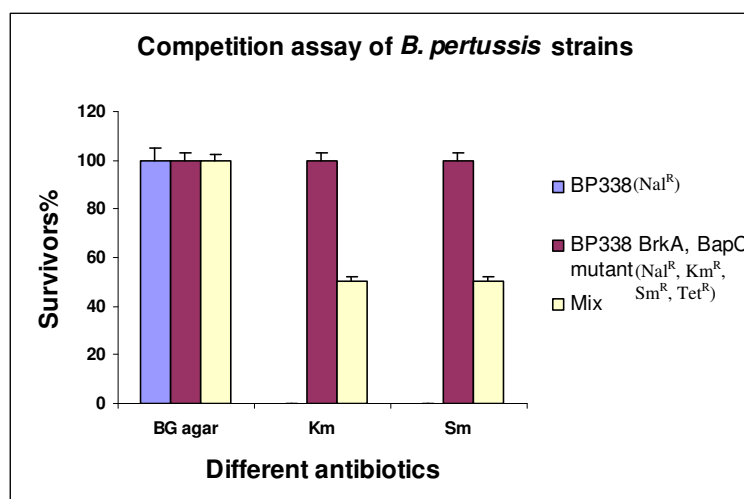


Fig. 3.44 Continued.

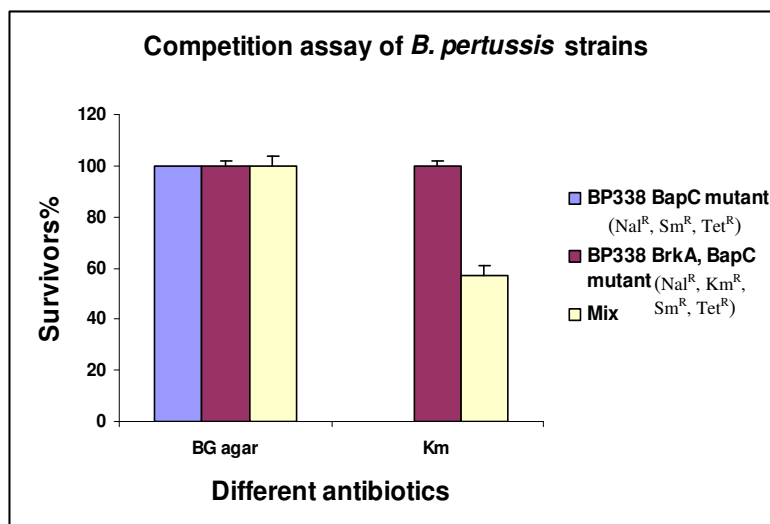
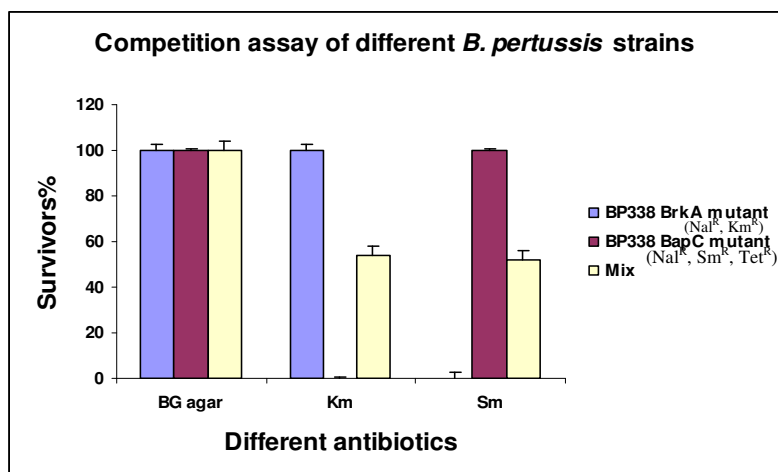
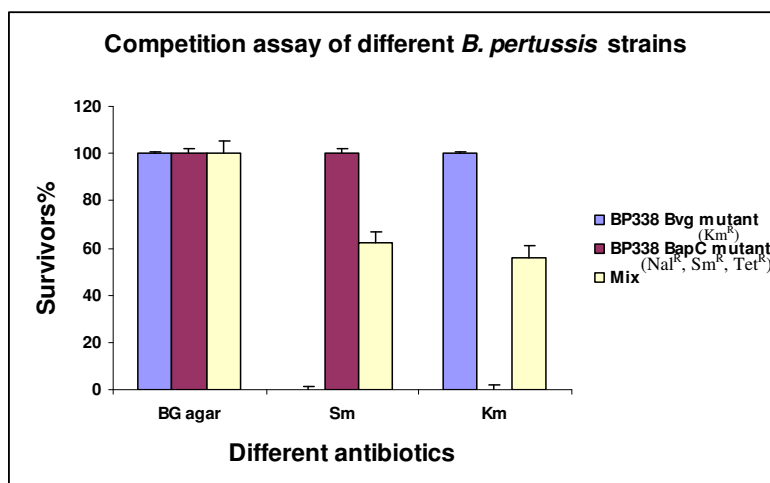
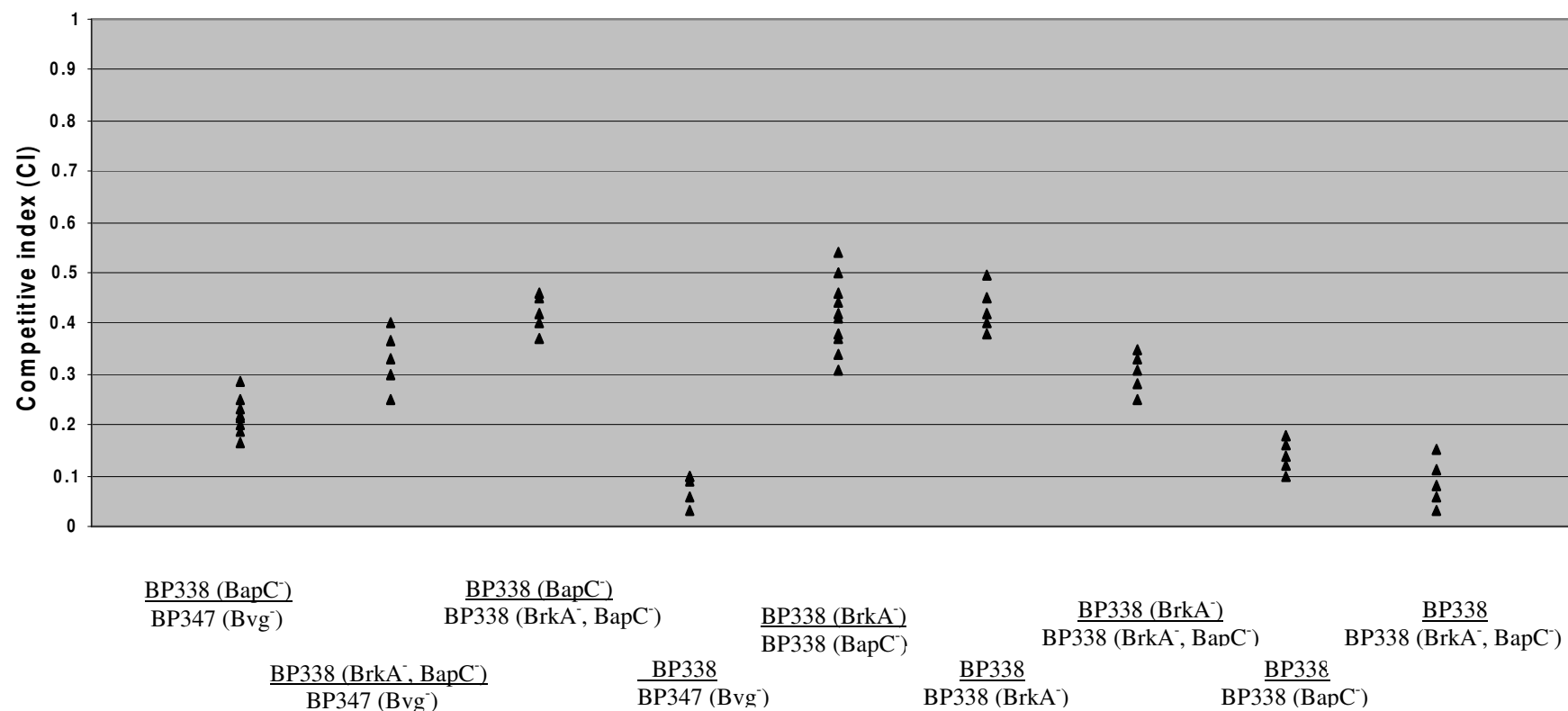


Figure 3.45 Competition assays of virulence for mice

For competition experiments, different strains of *B. pertussis* were mixed in a 1:1 ratio and 3 week old mice were infected intranasally. At 7 days, lung counts were used to determine the output of strain 1 CFU compared to the strain 2 CFU by selection on appropriate antibiotic plates. *In vivo* competitive indices (CI) for each mouse were calculated as:

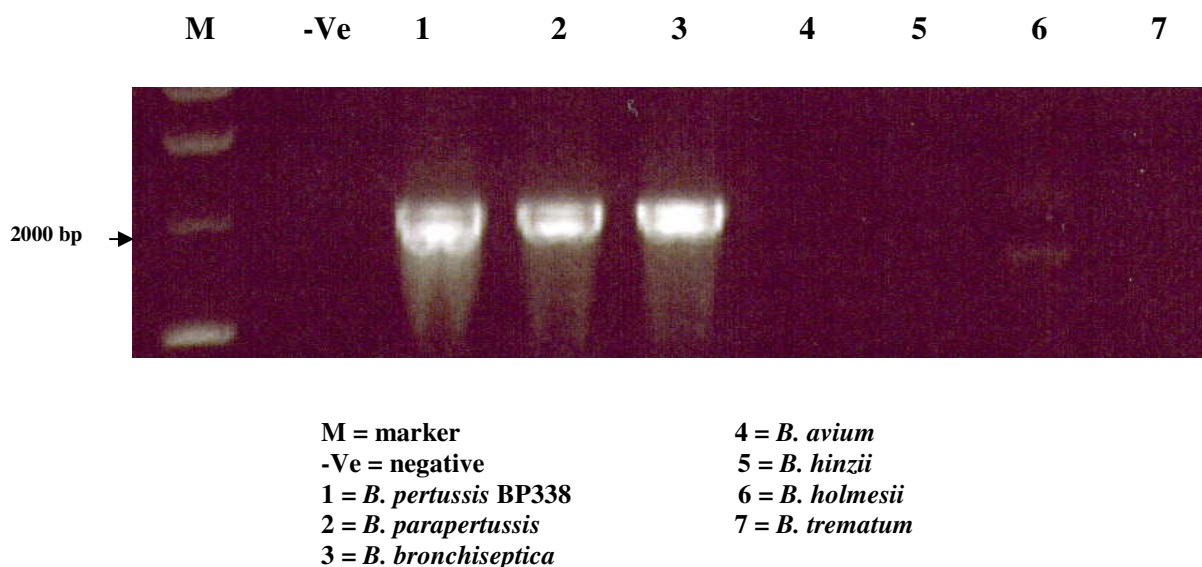
$CI = 1 / (\text{ratio of strain 1 to strain 2 output CFU} / \text{ratio of strain 1 to strain 2 input CFU})$.



3.18 Identification of the *bapC* locus in other *Bordetella* species

To identify the presence of the *bapC* locus in other *Bordetella* species, PCR reactions with *bapC* primers were performed initially. Forward and reverse primers (BAP5F, BAP5R), which amplify most of the *bapC* gene in *B. pertussis* Tohama I, were utilised and PCR was performed on chromosomal DNA extracted as described in section 2.2.1. Many attempts were carried out to amplify the *bapC* fragment in other bordetellae using different PCR parameters and CaCl₂ or Q-solution concentrations. Fig. 3.46 shows that the *bapC* gene (2.2 kbp) was amplified in *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* strains only, and no evidence was obtained of any bands in *B. avium*, *B. hinzii*, *B. holmesii* or *B. trematum* species.

Figure 3.46 Agarose gel (0.7%) showing the PCR products of *Bordetella* genomic DNAs using forward and reverse *bapC* primers



PCR was performed on chromosomal DNA extracted as described in section 2.2.1, using primers *bapC* forward and reverse (BAP5F, BAP5R) which amplify most of the *bapC* gene.

Another attempt, using the Southern blot method, was performed to detect the *bapC* locus or homologous sequences in other *Bordetella* species. An amplified digoxigenin-labelled probe of the *bapC* gene (section 2.10.1) was used. Southern blots containing *Nde*I, *Bgl*II or *Xho*I-digested chromosomal DNA from *B. pertussis* (BP338), *B. parapertussis*, *B. bronchiseptica*, *B. avium*, *B. hinzii*, *B. holmesii* and *B. trematum* were probed with a labelled fragment of *bapC* gene. To overcome the low yield of Dig-labeled PCR product and hybridization smearing, PCR parameters were optimized after many attempts by adding Q-solution and reducing the concentration of DIG-dUTP and template. DIG-dUTP nucleotides in the template slow the polymerase and eventually reduce the ability of the polymerase to synthesize full length products that consist of primer sequences required for the next amplification round. The optimal amount of template used was 10 ng genomic DNA and for the probe 0.5 µl of labelled PCR product was used per ml hybridization buffer. High background was the other problem that was overcome, to some extent, by preheating the wash buffers, reducing the probe concentration and using high stringency buffer.

Bordetella genomic DNAs were digested with *Nde*I, *Bgl*II or *Xho*I and analysed by agarose gel electrophoresis (Figs. 3.47A, B and C). Figures 3.48A, B and C show the result of Southern blotting analysis of the DNA with the *bapC* probe. Strong bands were observed in lanes 1, 2 and 3 which imply hybridisation to the genomes of *B. pertussis*, *B. pertussis* and *B. parapertussis*. No evidence of the *bapC* locus or true orthologues was found in any of the other *Bordetella* species.

Figure 3.47 *Bordetella* genomic DNAs digested with *Xho*I (a), *Bgl*II (b) and *Nde*I (c) enzymes and analysed by gel electrophoresis (0.7% agarose)

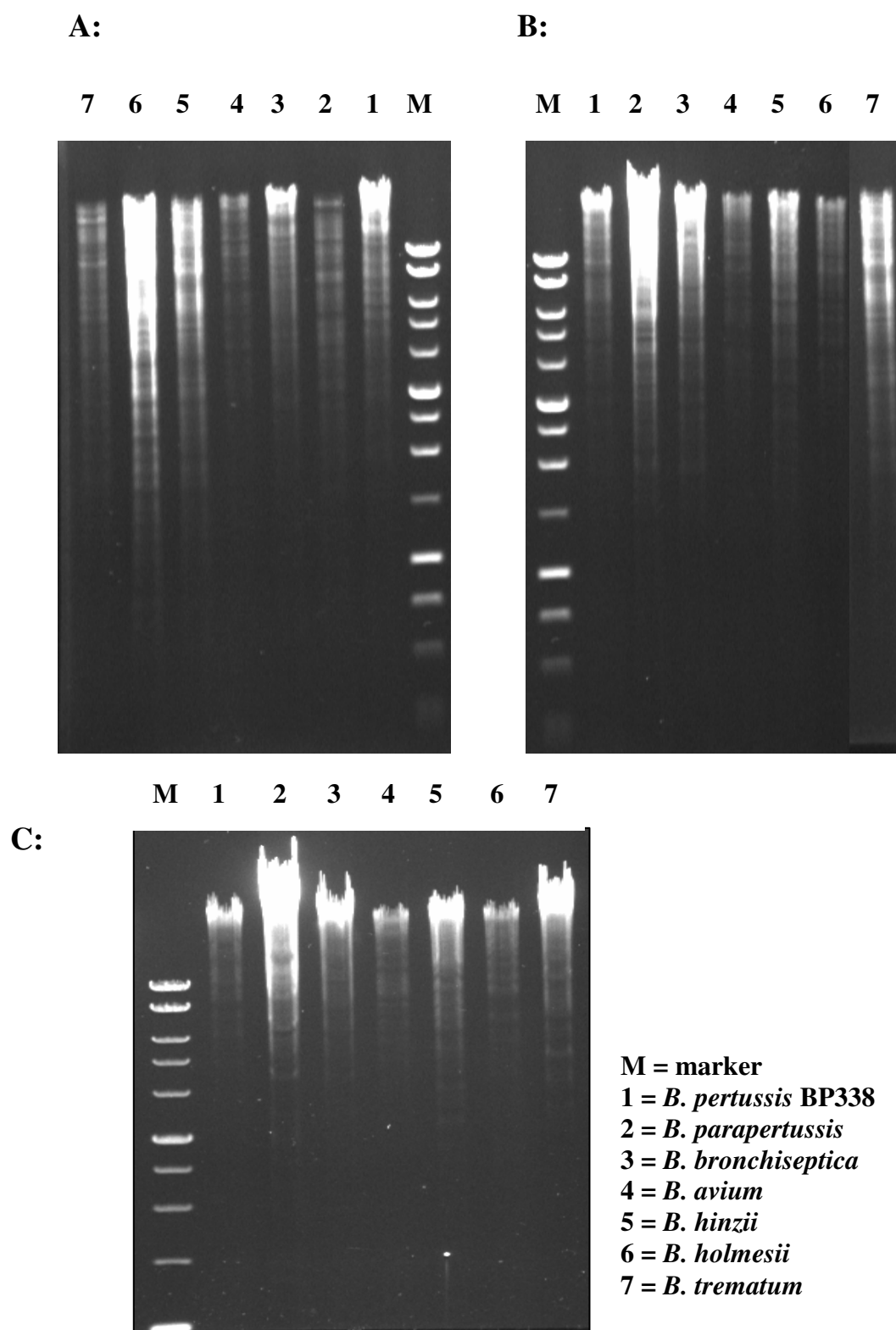
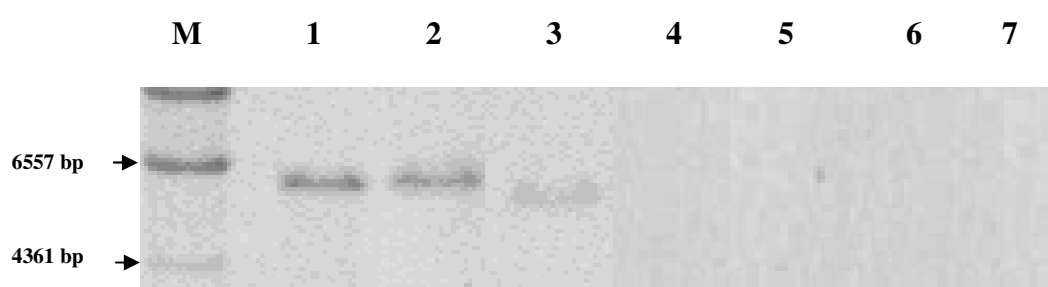


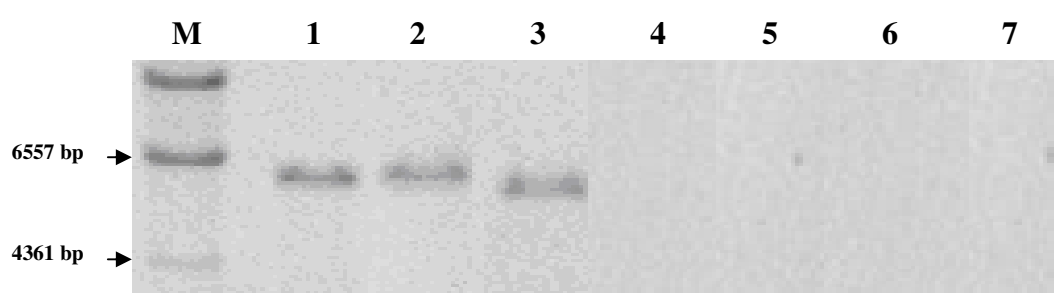
Figure 3.48 Southern blot analysis of *Bordetella* genomic DNAs, digested with *Xho*I (A), *Bgl*II (B), and *Nde*I (C), with *bapC* probe

Chromosomal DNAs were isolated, digested with *Nde*I, *Bgl*II and *Xho*I and separated on a 0.7% agarose gel. A blot was probed with labelled DNA from the *bapC* locus. DNA markers (in base pairs) are indicated on the gel. All samples were run on the same gel and probed on the same membrane. The image is a composite to represent spatially disparate bands.

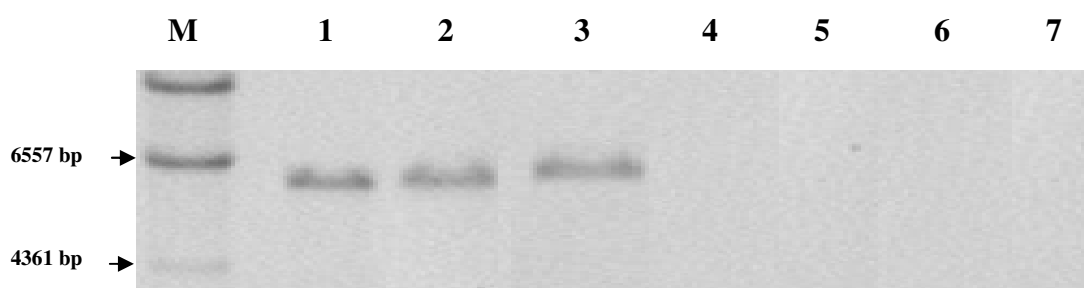
A:



B:



C:



M = Marker
 1 = *B. pertussis*
 2 = *B. parapertussis*
 3 = *B. bronchiseptica*

4 = *B. avium*
 5 = *B. hinzii*
 6 = *B. holmesii*
 7 = *B. trematum*

3.19 Prediction of a signal sequence in the BapC protein of *Bordetella* species

Bioinformatics is concerned with the computational prediction of structural and functional properties of genes and the proteins they encode, based on their nucleotide and amino acid sequences. Since one of the crucial properties of a protein is its subcellular location, prediction of protein sorting is an important question in bioinformatics. A fundamental distinction in protein sorting is that between secretory and non-secretory proteins, determined by a cleavable N-terminal sorting signal, the secretory signal peptide.

SignalP is the most popular software for prediction of classically-secreted proteins. In version 3, correctness of the cleavage site predictions have increased notably for all three organism groups, eukaryotes, Gram-negative and Gram-positive bacteria. SignalP consists of two different predictors based on neural network and hidden Markov model algorithms. The signal prediction server has been developed a similar procedure based on pair-wise alignments for sequences with functional sites.

3.19. 1 Description of the scores

The graphical output from SignalP (neural network) comprises three different scores, *C*, *S* and *Y*. Two additional scores are reported in the SignalP3-NN output, namely the *S-mean* and the *D-score*.

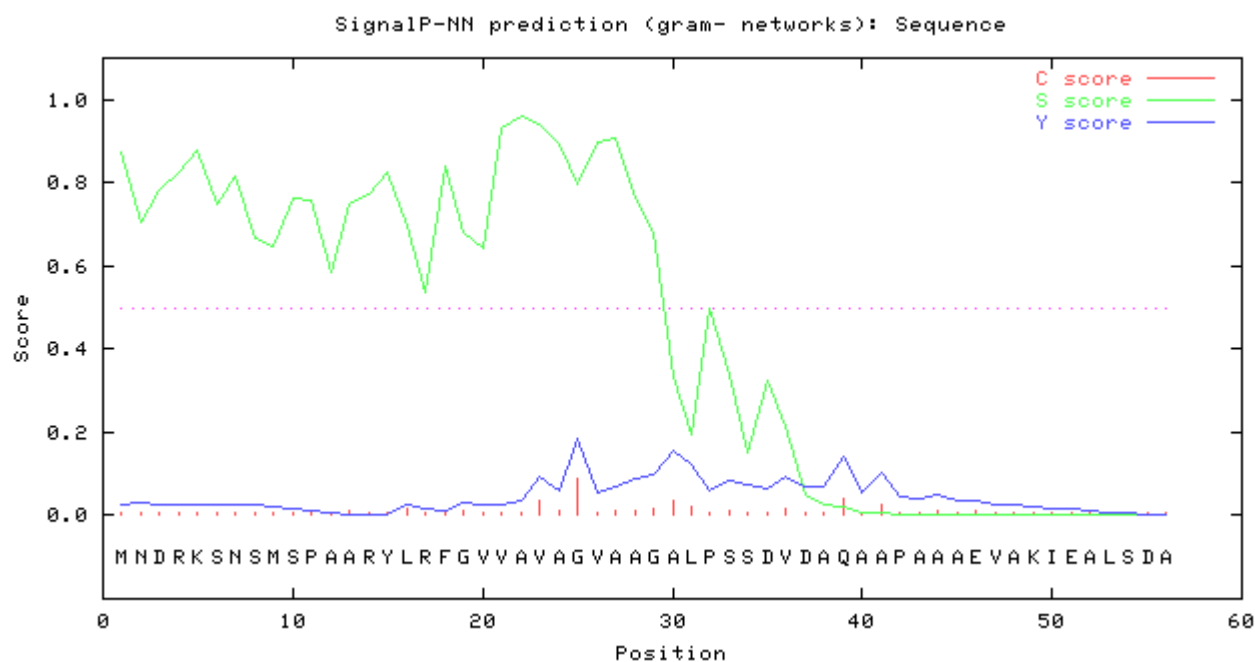
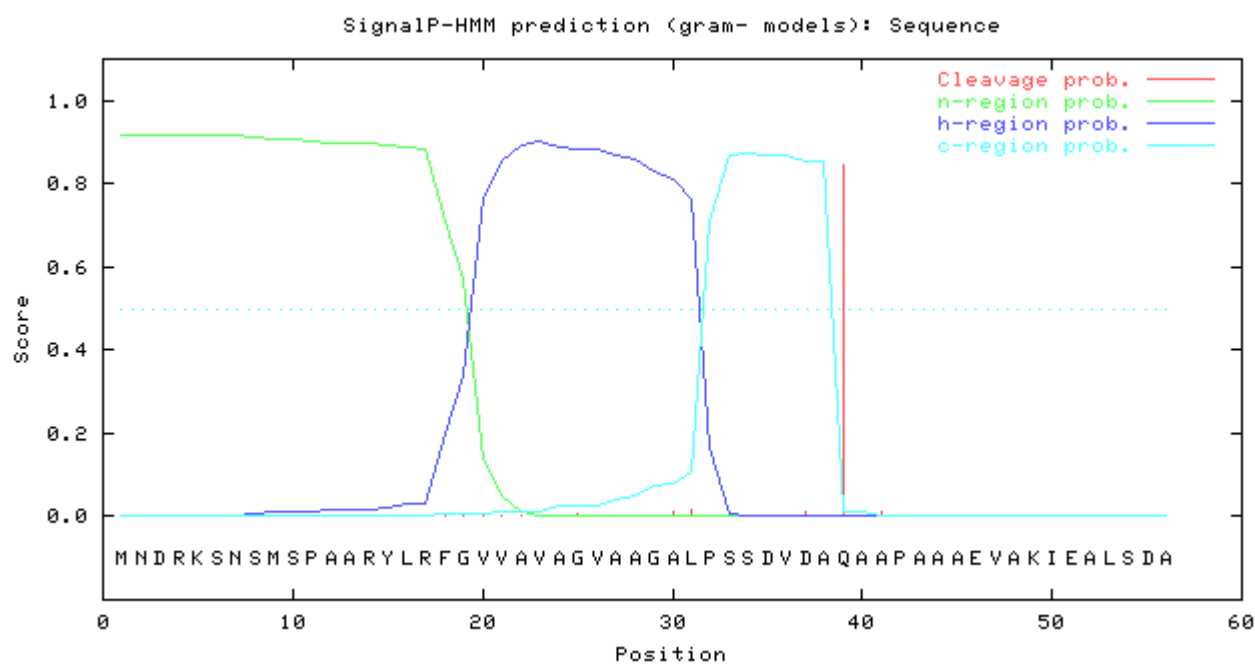
For each organism class in SignalP; Eukaryote, Gram-negative and Gram-positive, two different neural networks are used, one for predicting the actual signal peptide and one for predicting the position of the signal peptidase I (SPase I) cleavage site. The *S-score* for the signal peptide prediction is reported for every single amino acid position in the submitted

sequence, with high scores indicating that the corresponding amino acid is part of a signal peptide, and low scores indicating that the amino acid is part of a mature protein.

The *C-score* is the “cleavage site” score. For each position in the submitted sequence, a *C-score* is reported, which should only be significantly high at the cleavage site. Confusion is often seen with the position numbering of the cleavage site. When a cleavage site position is referred to by a single number, the number indicates the first residue in the mature protein, meaning that a reported cleavage site between amino acid 26-27 means that the mature protein starts at position 27. *Y-max* is a derivative of the *C-score* combined with the *S-score* resulting in a better cleavage site prediction than the raw *C-score* alone. This is due to the fact that multiple high-peaking *C-scores* can be found in one sequence, where only one is the true cleavage site.

The cleavage site is assigned from the *Y-score* where the slope of the *S-score* is steep and a significant *C-score* is found. The *S-mean* is the average of the *S-score*, ranging from the N-terminal amino acid to the amino acid assigned with the highest *Y-max* score, thus the *S-mean* score is calculated for the length of the predicted signal peptide. The *S-mean* score was used in SignalP version 2.0 as the criterion for discrimination of secretory and non-secretory proteins. The *D-score* is introduced in SignalP version 3.0 and is a simple average of the *S-mean* and *Y-max* score. The score shows superior discrimination performance of secretory and non-secretory proteins to that of the *S-mean* score which was used in SignalP version 1 and 2. For non-secretory proteins all the scores represented in the SignalP3-NN output should ideally be very low. The hidden Markov model calculates the probability of whether the submitted sequence contains a signal peptide or not. Furthermore, the cleavage site is assigned by a probability score together with scores for the n-region, h-region, and c-region of the signal peptide, if such one is found.

At the start of this project, no signal sequence had been identified for BapC from *B. pertussis* Taberman W/T strain. Available genome sequences and BapC protein sequences of *B. pertussis* Tohama I (locus_tag BP2738), *B. parapertussis* (locus_tag BPP2591) and *B. bronchiseptica* (locus_tag BB2033) allowed prediction of signal sequences. Analysis of BapC protein in *B. pertussis* (Fig. 3.49), *B. parapertussis* (Fig. 3.50) and *B. bronchiseptica* (Fig. 3.51) with signalP 3.0 server showed a signal peptide of 24 amino acids by the Neural Network prediction method (A) and a maximum cleavage site probability at 38 amino acids by the Hidden Markov Model method (B) for *B. pertussis* (Fig. 3.49), and a signal peptide of 29 amino acids by the neural Network prediction method and a maximum cleavage site probability at 38 amino acids by the hidden Markov Model for both *B. parapertussis* (Fig. 3.50) and *B. bronchiseptica* (Fig. 3.51).

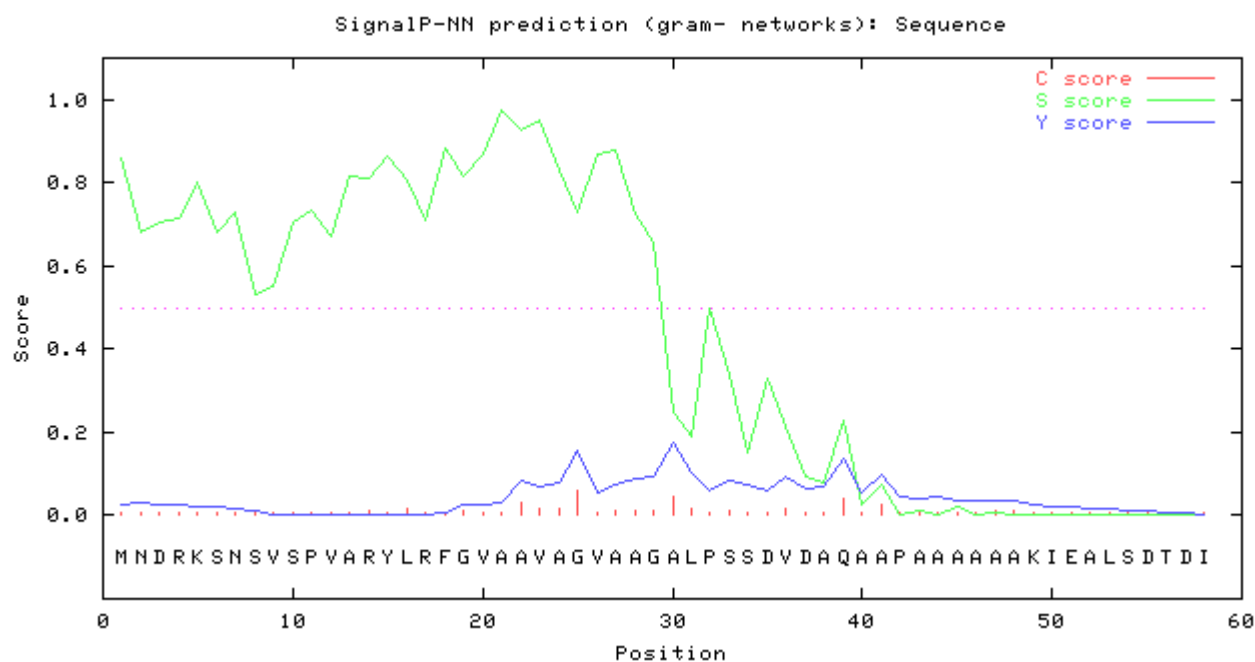
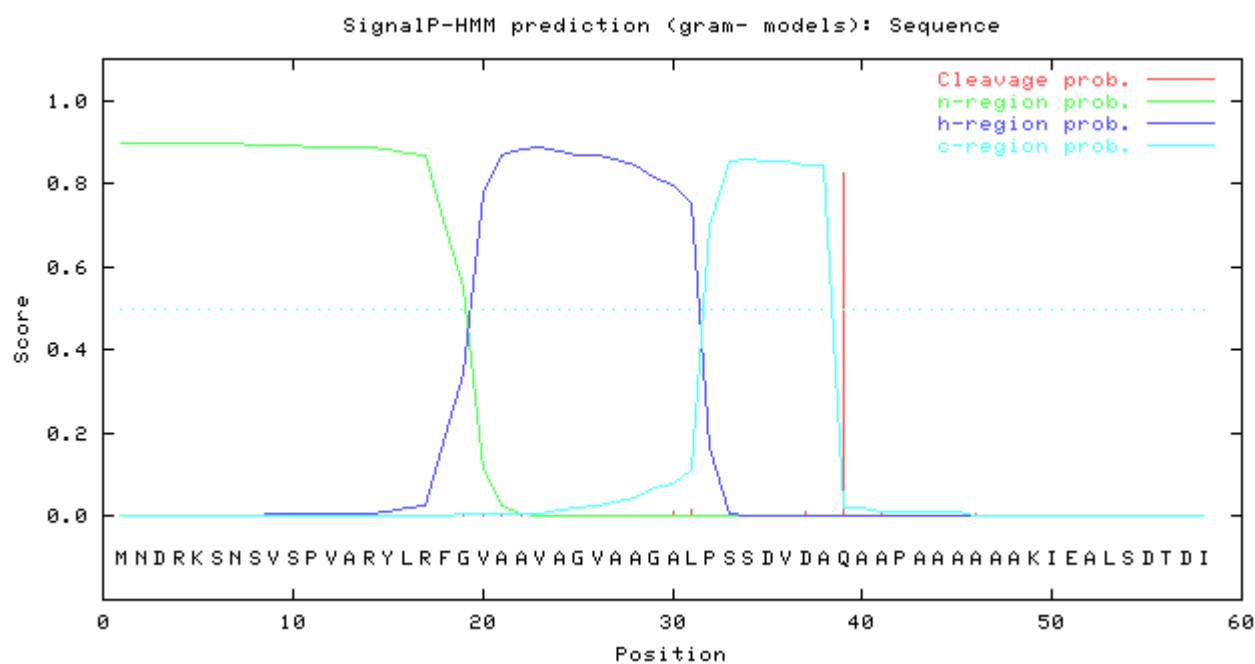
Figure 3.49 SignalP-NN result of BapC in *Bordetella pertussis***A:****B:**

Measure	Position	Value	Cutoff	signal peptide?
max. C	25	0.090	0.52	NO
max. Y	25	0.185	0.33	NO
max. S	22	0.959	0.92	YES
mean S	1-24	0.772	0.49	YES
D	1-24	0.478	0.44	YES

Most likely cleavage site between pos. 24 and 25: AVA-GV

Prediction: Signal peptide; Signal peptide probability: 0.917

Max cleavage site probability: 0.844 between pos. 38 and 39

Figure 3.50 SignalP-NN result of BapC in *Bordetella parapertussis***A:****B:**

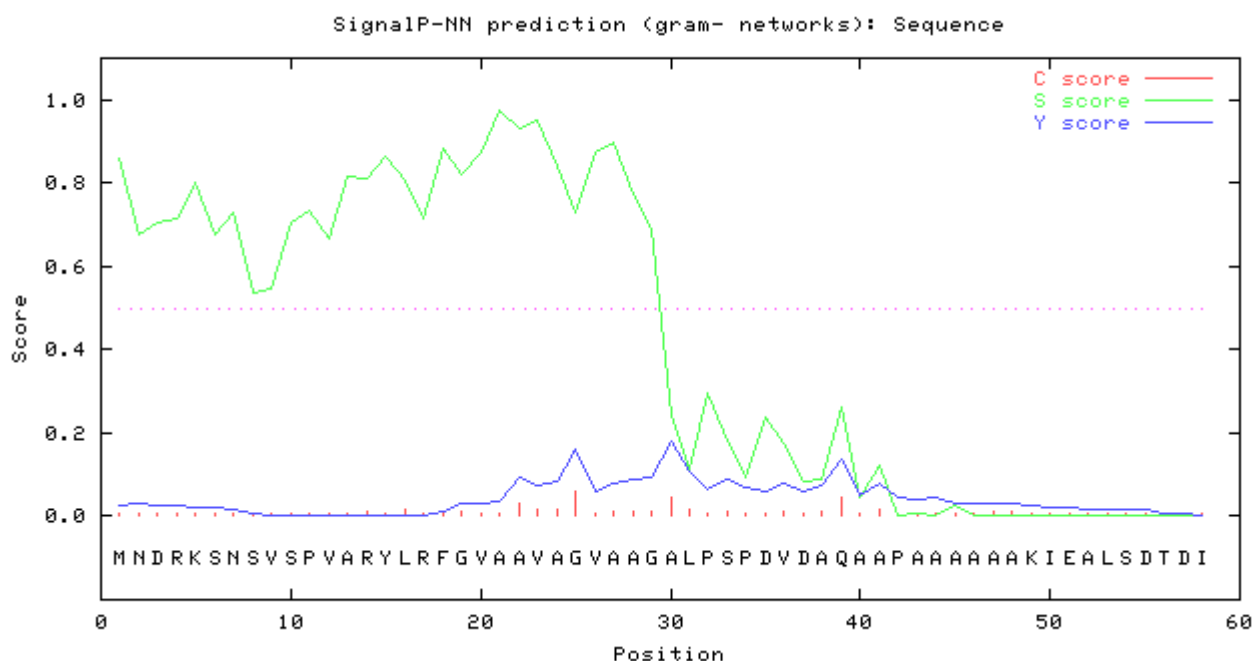
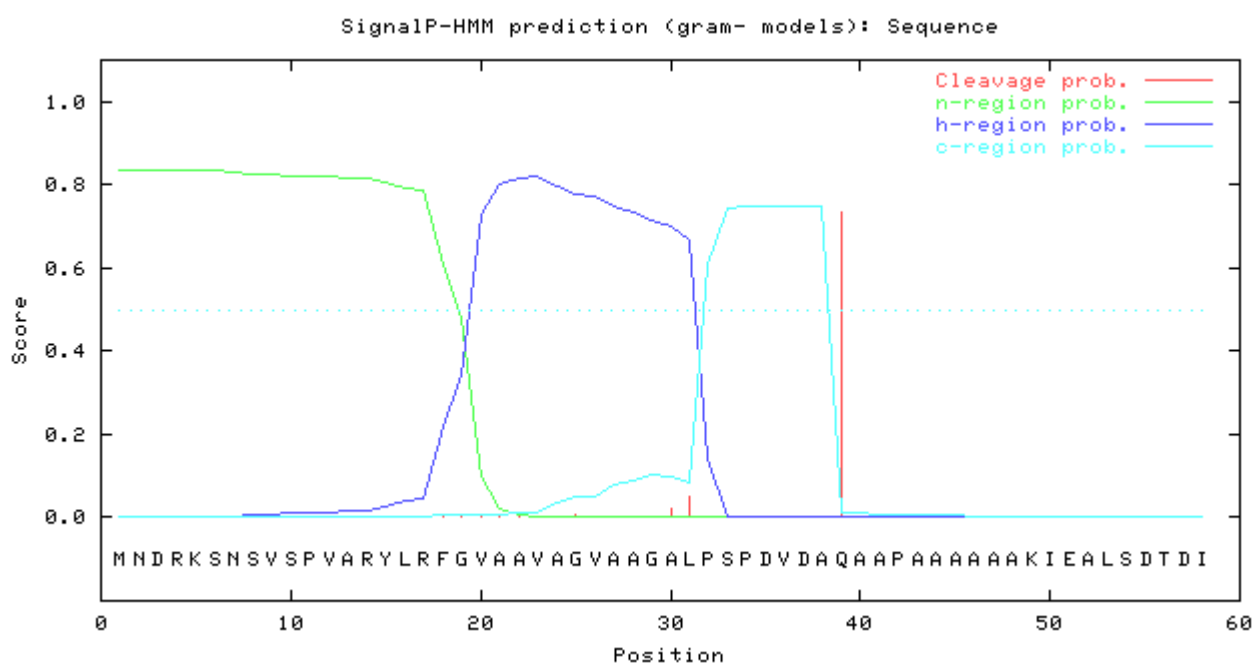
Measure	Position	Value	Cutoff	signal peptide?
max. C	25	0.059	0.52	NO
max. Y	30	0.174	0.33	NO
max. S	21	0.973	0.92	YES
mean S	1-29	0.775	0.49	YES
D	1-29	0.475	0.44	YES

Most likely cleavage site between pos. 29 and 30: AAG-AL

Prediction: Signal peptide

Signal peptide probability: 0.897

Max cleavage site probability: 0.825 between pos. 38 and 39

Figure 3.51 SignalP-NN result of BapC in *Bordetella bronchiseptica***A:****B:**

Measure	Position	Value	Cutoff	signal peptide?
max. C	25	0.059	0.52	NO
max. Y	30	0.180	0.33	NO
max. S	21	0.975	0.92	YES
mean S	1-29	0.779	0.49	YES
D	1-29	0.479	0.44	YES

Most likely cleavage site between pos. 29 and 30: AAG-AL

Prediction: Signal peptide

Signal peptide probability: 0.833

Max cleavage site probability: 0.735 between pos. 38 and 39

3.20 Cloning and expression of BapC with and without a signal sequence

It was of interest to investigate the role of the BapC protein, for example in serum sensitivity, away from the other autotransporter proteins of *B. pertussis* by directionally ligating the whole *bapC* gene with and without signal sequence (amino acids 1-38) into an expression vector pET33b (+) (Novagen) (Appendix II.2) for high level expression in *E. coli* BL21 (DE3) and BL21 (DE3) pLysS (Table 2.1). The latter was used to reduce basal (leaky) expression of their recombinant gene. This would make purified recombinant protein available for further study.

The vector was separately digested with *NdeI/BamHI* or *NheI/BamHI* and bands of the expected size, 5383 bp, were obtained, shown in Fig. 3.52. For vector preparation, the restriction enzyme manufacturer's recommended buffer and incubation conditions were used. In accordance with the manufacturer's instruction, when using compatible buffers and those whose sites are more than 10 bp apart the two enzymes could be used together in the same reaction. Unfortunately, after many attempts, these enzymes displayed "star activity" or poor cutting. Digestion was therefore performed sequentially; with the first digestion using the enzyme that was the poorest cutter and then the second enzyme added after the digestion had been verified, by running a sample of the reaction on an agarose gel and gel extraction (section 2.3.4). To decrease the non-recombinant background caused by religation of the digested vector, which was undetectable by gel analysis, the cut vector was dephosphorylated with calf intestinal alkaline phosphatase. Following digestion, the vector was gel-purified (section 2.3.4) before insert ligation to remove residual supercoiled plasmids in order to reduce the effort required to screen for the correct constructs.

For amplification of DNA encoding the *bapC* gene without the signal sequence, an ATG start codon was engineered into the 5' end of the artificial open reading frame, once the *NheI*

restriction had been incorporated into primers, to permit translation (BAPCF25) (Table 2.5). Primers were designed according to the *bapC* sequence of the *B. pertussis* Tohama genome sequence (locus_tag BP2738) and a *B. pertussis* BP338 genomic DNA was used as template. Restriction sites such as *NdeI* (BAPCF14 and BAPCF24) or *BamHI* (BAPCR12) were incorporated into the primers (Table 2.5) to allow directional cloning into the expression vector. High fidelity *Pfu* DNA polymerase enzyme, a limited number of PCR cycles and high concentrations of primers and target DNA were utilised to minimize the potential for mutation due to errors generated by the PCR reaction.

The amplified DNAs encoding the *bapC* gene with or without signal sequence were subjected to restriction digestion with either *NdeI/BamHI* or *NheI/BamHI* as appropriate and then gel-purified. Figs 3.53a and b show bands of the expected size, 2981 bp and 2870 bp for *bapC* gene with and without signal sequence, respectively. Ligations were performed according to section 2.5.2 and products transformed by electroporation into either *E. coli* BL21 (DE3) or BL21 (DE3) pLysS (Table 2.1) competent cells, as appropriate. The resultant colonies were screened for the presence of insert of the correct size, either directly with colony PCR (Fig. 3.54) (section 2.8.4) or after plasmid extraction and digestion with the enzymes used to generate the cohesive ends prior to ligation (Fig. 3.55). Fig. 3.54 shows the results obtained from the clones that were selected because they showed the inserts of the appropriate size. Lanes 1 and 2 show the expected band of 2981 bp after amplification of the *bapC* gene with signal sequence, and lanes 3 and 4 show the expected size of 2870 bp after amplification of the *bapC* gene without signal sequence. Those containing an insert of correct size were subjected to sequencing (section 2.9) and then aligned with the genome sequence (data not shown).

Figure 3.52 Agarose gel (0.7%) showing purified digested pET33b vector.

Restriction digestion of pET33b vector was performed using two sets of enzymes (*NheI/BamHI* or *NdeI/BamHI*) and then purified.

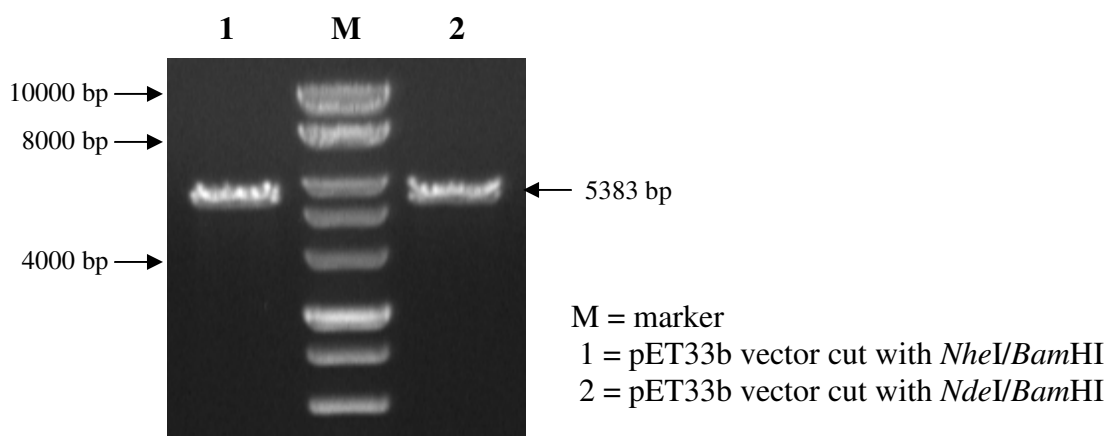
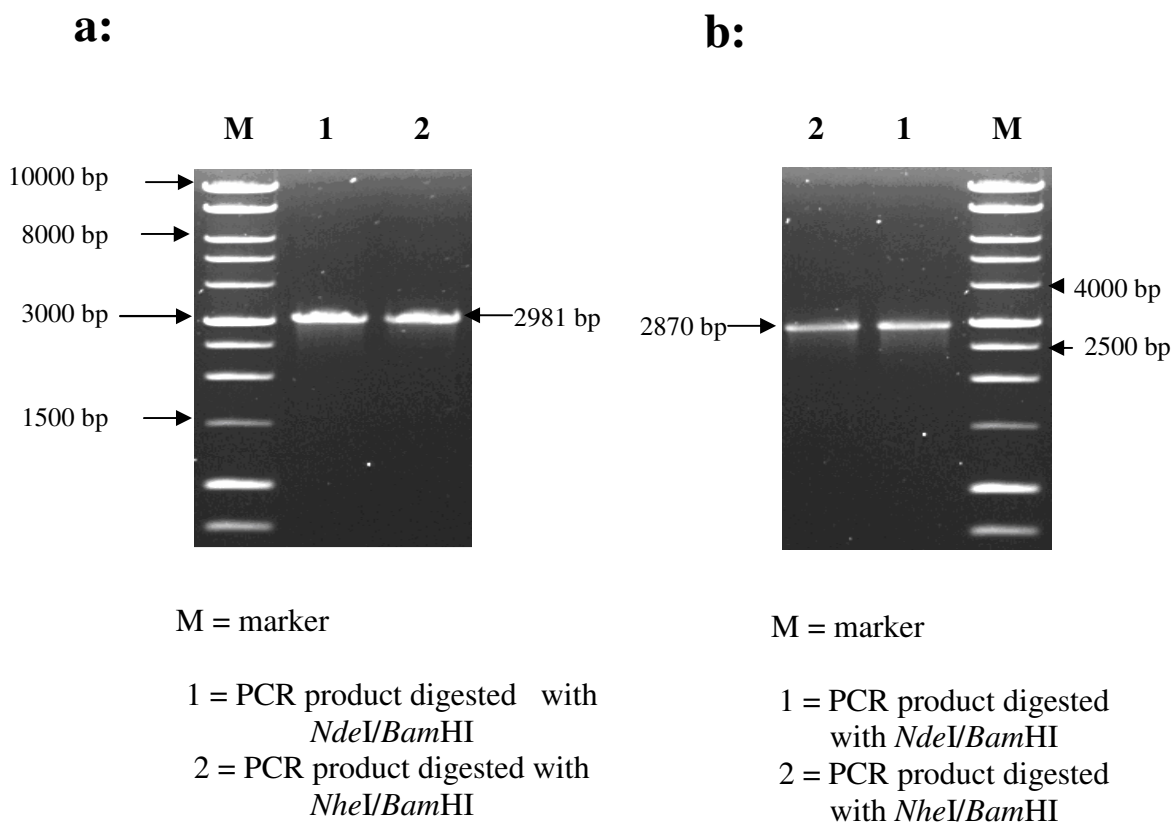
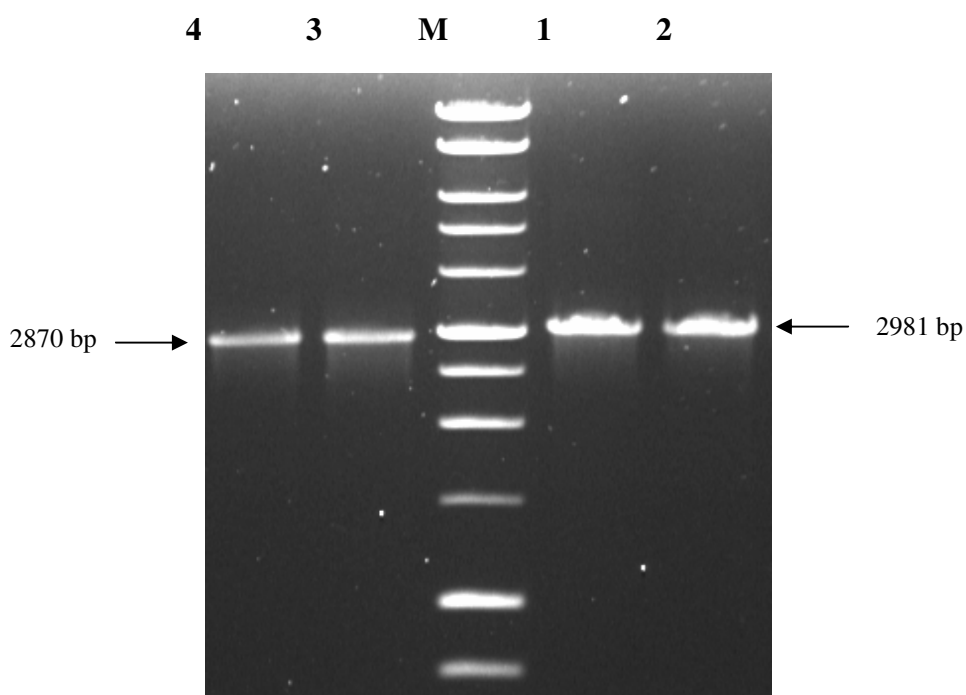
**Figure 3.53 Agarose gel (0.7%) showing digested and purified PCR products of *bapC* gene with signal sequence (a) and digested purified nested gradient PCR products of *bapC* gene without signal sequence (b).**

Figure 3.54 Agarose gel (0.7%) showing PCR products of selected clones using *bapC* primers after plasmid purification



M = marker

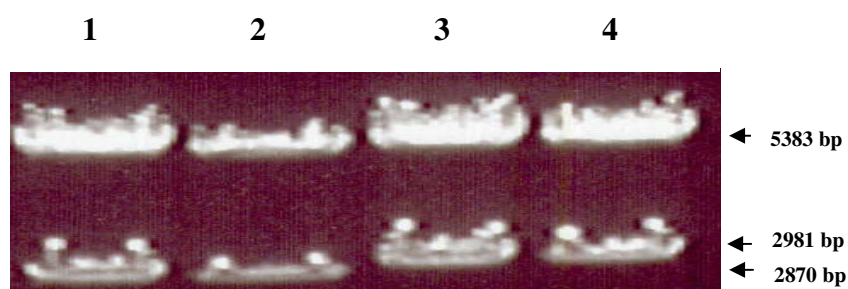
1 = PCR product using primers BAPCF14, BAPCR12

2 = PCR product using primers BAPCF15, BAPCR12

3 = PCR product using primers BAPCF24, BAPCR12

4 = PCR product using primers BAPCF25, BAPCR12

Figure 3.55 Agarose gel (0.7%) showing purified plasmid from selected clones digested with *NheI/BamHI* and *NdeI/BamHI* enzymes



1 = pET33b (BapC - SS) digested with *NdeI/BamHI*

2 = pET33b (BapC - SS) digested with *NheI/BamHI*

3 = pET33b (BapC + SS) digested with *NdeI/BamHI*

4 = pET33b (BapC + SS) digested with *NheI/BamHI*

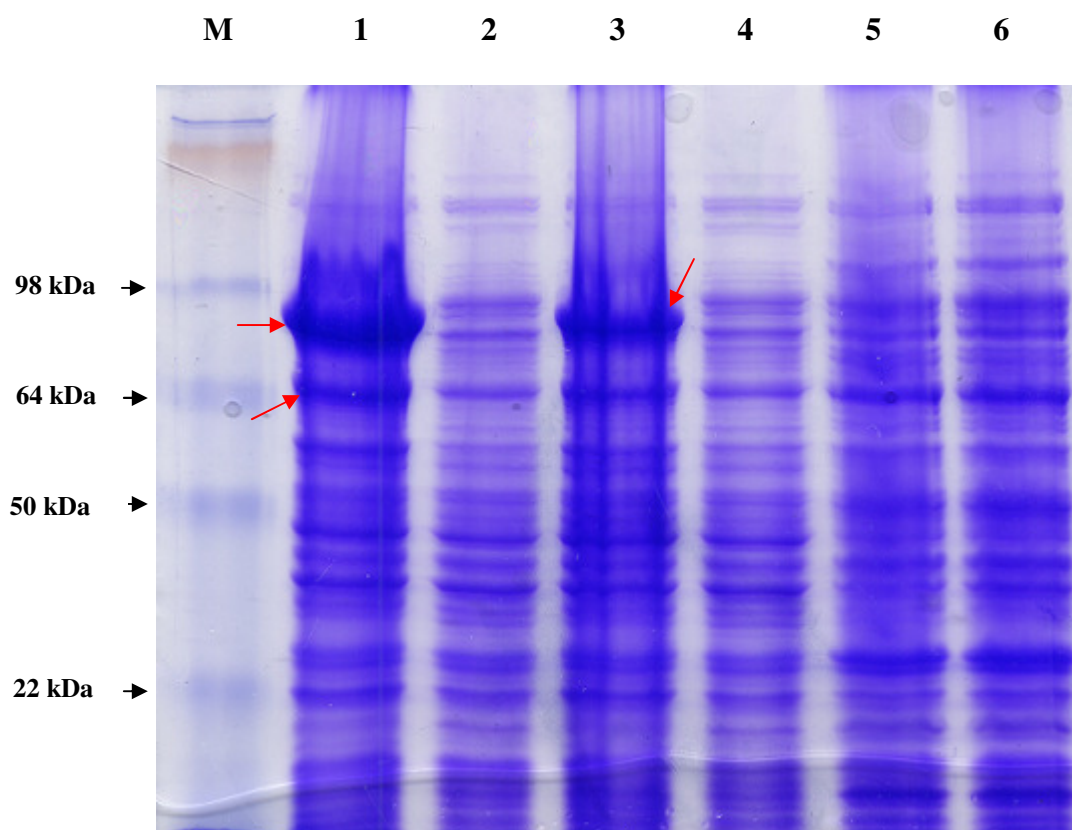
3.20.1 Sequencing of cloned *bapC* and aligning with the *bapC* in database

Automated sequencing (section 2.9) was performed on pET33b (BapC - SS) and pET33b (BapC + SS) vectors by the Molecular Biology Support Unit (MBSU). As the accurate average read length was around 400-600, the plasmids were sequenced in both directions to allow more sequences to be obtained for analyses. The resultant sequences derived from electropherograms were analyzed and edited manually using Chromas (version 1.45) and BioEdit version 5.0.6 and then aligned with the *bapC* gene of *B. pertussis* Tohama in the database using BLAST2 Sequences. The sequence alignments for both constructs showed no difference in both forward and reverse directions compared to the *bapC* gene in the database (data not shown).

3.20.2 Overexpression of BapC protein in *E. coli*

After the pET33b plasmids were established in *E. coli* BL21 (DE3) and BL21 (DE3) pLysS strains, expression of the *bapC* DNA was induced by the addition of IPTG to cultures growing in LB. Similar results were obtained with both strains and only the results for BL21 (DE3) pLysS will be described. In brief, a single colony was picked off from a freshly streaked plate and inoculated into 5 ml of LB containing appropriate antibiotics (34 µg/ml of chloramphenicol for BL21 (DE3) pLysS) and grown overnight at 25°C-30°C. One ml of this culture was then transferred into 100 ml of LB (section 2.11.2). Once the OD_{600nm} had reached 0.5-0.6, a range of IPTG concentrations from 0.1 to 1 mM were added to establish the optimal IPTG concentration at 25°C-30°C to maximise the yield of the recombinant protein. The induced cultures were examined for target protein expression using SDS-PAGE and Western blotting. Just prior to induction, the 100 ml culture was split into 2×50 ml cultures, and 1 mM final concentration of IPTG added to one of the 50 ml cultures and the other used as an un-induced control. In all cases, BapC protein expression was tightly regulated and was not detectable from uninduced cultures in either a Coomassie-blue stained

Figure 3.56 Protein profile showing the whole-cell fractions of *E. coli* BL21 (DE3) pLysS containing pET33b (+) vector expressing BapC protein with or without the signal sequence



M = marker

1 = *E. coli* pET33b (BapC + SS) + IPTG

2 = *E. coli* pET33b (BapC + SS) - IPTG

3 = *E. coli* pET33b (BapC - SS) + IPTG

4 = *E. coli* pET33b (BapC - SS) - IPTG

5 = *E. coli* pET33b + IPTG

6 = *E. coli* pET33b - IPTG

The figure shows the whole-cell protein profiles by SDS-PAGE (10%) of the *E. coli* strains grown in LB medium, with and without induction with 1 mM IPTG.

gel (Fig. 3.56) or, where performed, immunoblots with anti-His₆ antibodies (data not shown).

Figure 3.56 shows the protein profiles of the whole-cell fractions of *E. coli* BL21 (DE3) pLysS expressing the full length BapC protein with signal sequence in induced (lane 1) and uninduced (lane 2) and without signal sequence in induced (lane 3) and uninduced (lane 4) conditions. Lanes 5 and 6 show protein profiles of *E. coli* control strains with the pET33b vector alone with no insert, in induced (lane 5) and uninduced (lane 6) conditions. The top arrowed band in lane 1 at ~90 kDa is presumed to represent either the unprocessed (104 kDa) or the processed (71 kDa) form of BapC. The second arrowed band in lane 1 that is slightly more prominent than in other lanes could possibly be the N-terminus or α -domain of BapC after cleavage since it is nearer to ~71 kDa, the molecular weight of the α -domain. The predicted molecular weight of the whole BapC (from the first ATG in signal sequence) and the N-terminal or passenger domain are about 104 and 71 kDa, respectively, using ProtParam tool (<http://www.expasy.org/tools/protparam.html>).

3.20.3 Identification of BapC protein expressed in *E. coli*

The identification of the BapC protein in *E. coli* expression strains was performed by manually excising the bands of interest (red arrows) from an SDS-PAGE gel and subjecting them to mass spectrometry (section 2.11.8). The peptide mass fingerprints obtained by MS were subjected to a database search by pasting the peptides into a protein database (Mascot) search tool which defines significant matches with known protein sequences (Appendix II.7a). The results showed that the ~90 kDa band in lane 1 in Fig. 3.56 corresponded to the unprocessed form of BapC protein published in the database (locus_tag BP2738) with 46% known-peptide coverage (Appendix II.7b), suggesting that the BapC in *B. pertussis* strain BP338 would indeed be expressed in this strain. However, the lower molecular weight band

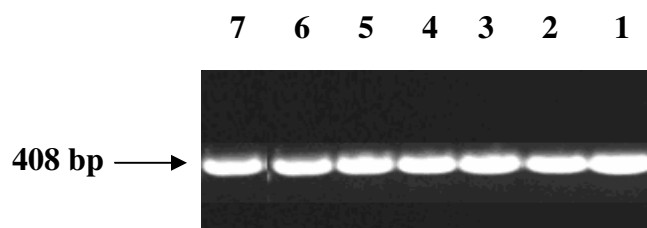
in lane 1 showed only slight identity to the α -domain of BapC with 14% known-peptide coverage.

3.21 Sequence determination of *bapC* gene

The gene in the genome sequence of *B. pertussis* strain Tohama encoding BapC protein was reported by Sebahia in 2006 (GenBank) to be a pseudogene (Accession No. NP_881344) with frameshifts in two homopolymeric tracts (HPT), one of 13 cytosine residues and less likely one of 11 guanine residues towards the 5' end of *bapC* (Appendix II.3). The consequences of these mutations alter the amino acid sequences and result in premature termination of the protein. In the present study with strain Tohama (section 3.3) and in the previous studies of Bokhari (2002) and Blackburn (2000), with strains BP Taberman and BP Tohama, evidence was presented that the BapC protein was in fact expressed. To address this anomaly, the *bapC* gene of different *B. pertussis* strains was sequenced using *bapC* forward and reverse primers (Table 2.5) amplifying two overlapping fragments of the 5'-end sequence of the *bapC* gene carrying either the poly(C) or poly(G) sequences to determine any variations in different *B. pertussis* strains and to compare them with the published Tohama I genome sequence.

B. pertussis strains were grown in CL medium (Appendix I) and genomic DNAs were isolated using standard procedures (section 2.2.1). PCR reactions were performed (section 2.8) using primer pairs MNF1/MNR1 and MNF2/MNR2 (Table 2.5) to amplify the regions containing the poly(C) or poly(G) sequences, respectively (Appendix II.3). Approximately 50 bp of overlapping sequence was generated between individual sequence reads to allow contigs to be assembled using Seqman II and BioEdit tools. DNA sequence determinations were performed by sending the excised and purified bands of the amplified PCR products from agarose gel electrophoresis to MBSU (Molecular Biology Support Unit) at Glasgow University for sequencing using the dideoxy method (section 2.9). The data were finally analysed via BioEdit tool, version 5.0.6.

Figure 3.57 Agarose gel (0.7%) showing the amplified fragment of *bapC* gene encompassing the poly(C) region, using primers MNF1, MNR1



M = marker

1 = BP338

2 = BP Tohama I

3 = BP Taberman

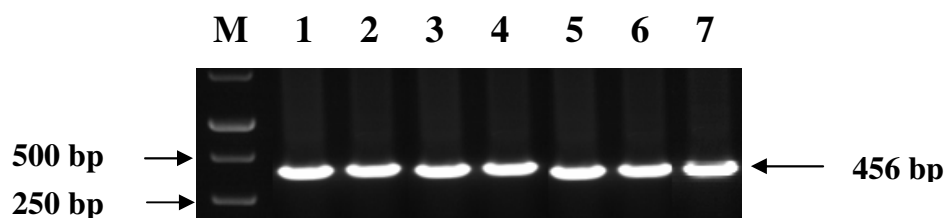
4 = BP PICU

5 = BP18323

6 = BP338 (BrkA⁻)

7 = BP Taberman (L1)

Figure 3.58 Agarose gel (0.7%) showing amplified fragment of *bapC* gene encompassing the poly(G) region, using primers MNF2, MNR2



M = marker

1 = BP338

2 = BP Tohama I

3 = BP Taberman

4 = BP PICU

5 = BP18323

6 = BP338 (BrkA⁻)

7 = BP Taberman (L1)

The PCR results (Fig. 3.57) using genomic DNA extracted from *B. pertussis* strains BP338 (a Tohama derivative), BP338 (BrkA⁻), BP Tohama I, BP Taberman, BP Taberman (L1) (mouse lung passaged strain), BP18323, BP PICU (Table 2.2), lanes 1-7 showed an expected approx. 408 bp of amplified fragment of the *bapC* gene downstream from the first ATG codon of the putative signal sequence, encompassing the poly(C) region.

An approximately 456 bp fragment that encompasses the poly(G) region of the *bapC* gene was also amplified from these six *B. pertussis* strains (Fig. 3.58) (lanes 1-7), using primers (MNF2/MNR2) which overlapped by 50 bp with the poly(C)-containing region to allow the two segments to be assembled as a continuous sequence.

3.21.1 Nucleotide sequence analysis of *bapC* gene region spanning the poly(C) and poly(G) tracts of different *B. pertussis* strains

Sequence analyses of amplicons were performed using software packages BioEdit and Seqman II. From dideoxynucleotide analyses, sequence data from forward and reverse primers were combined and aligned using CLUSTALW [<http://www.ebi.ac.uk/clustalw>] and MUSCLE (Multiple Sequence Comparison by Log-Expectation) [<http://www.ebi.ac.uk/muscle>] tools.

Analysis of the region spanning the poly(C) sequence in *B. pertussis* strains exhibited a polymorphism varying from 13-15 C nucleotides (Fig. 3.59). *B. pertussis* strains BP Taberman (I), BP338 (BrkA⁻), BP338, BP PICU and BP Tohama I (Glasgow strain) showed no difference in the number of C nucleotides compared to the BP Tohama genome sequence published in the database (without the frameshifts). *B. pertussis* strains BP Taberman (L1) and BP18323 exhibited 14 and 15 C nucleotides in poly(C) variable segment, respectively.

Figure 3.59 CLUSTAL W (1.83) multiple sequence alignment of 5'-end of *bapC* gene encompassing poly(C) sites

```

BP18323      -----GCGGCGAGATACTTGCCTTTTCGGCGTTGTCGCGGTAGCG 39
BP TabermanI -----GCGGCGAGATACTTGCCTTTTCGGCGTTGTCGCGGTAGCG 39
BP338BrkA-   AAATCCAATAGCATGTGCGCAAGCGGCGAGATACTTGCCTTTTCGGCGTTGTCGCGGTAGCG 60
BP338        -----TCGCCAGCGGCGAGATACTTGCCTTTTCGGCGTTGTCGCGGTAGCG 45
BP TabermanL1 -----GCGGCGAGATACTTGCCTTTTCGGCGTTGTCGCGGTAGCG 39
BPPICU       -----CCAGCGGCGAGATACTTGCCTTTTCGGCGTTGTCGCGGTAGCG 42
BP TohamaI    -----GCGGCGAGATACTTGCCTTTTCGGCGTTGTCGCGGTAGCG 39
                *****

BP18323      GGTGTAGCGGCGGGCGCGCTGCCTTCGTCGGAATGTCGATGCCAGGCCGCGCCGCCGCC 99
BP TabermanI  GGTGTAGCGGCGGGCGCGCTGCCTTCGTCGGAATGTCGATGCCAGGCCGCGCCGCCGCC 99
BP338BrkA-   GGTGTGGCGGCGGGCGCGCTGCCTTCGTCGGAATGTCGATGCCAGGCCGCGCCGCCGCC 120
BP338        GGTGTGGCGGCGGGCGCGCTGCCTTCGTCGGAATGTCGATGCCAGGCCGCGCCGCCGCC 105
BP TabermanL1 GGTGTGGCGGCGGGCGCGCTGCCTTCGTCGGAATGTCGATGCCAGGCCGCGCCGCCGCC 99
BPPICU       GGTGTGGCGGCGGGCGCGCTGCCTTCGTCGGAATGTCGATGCCAGGCCGCGCCGCCGCC 102
BP TohamaI    GGTGTGGCGGCGGGCGCGCTGCCTTCGTCGGAATGTCGATGCCAGGCCGCGCCGCCGCC 99
                *****

BP18323      GCCGAGGTAGCCAAGATCGAGGCTCTGTGCGATGCGGACATTTACAGCGACTACGAGCAC 159
BP TabermanI  GCCGAGGTAGCCAAGATCGAGGCTCTGTGCGATGCGGACATTTACAGCGACTACGAGCAC 159
BP338BrkA-   GCCGAGGTAGCCAAGATCGAGGCTCTGTGCGATGCGGACATTTACAGCGACTACGAGCAC 180
BP338        GCCGAGGTAGCCAAGATCGAGGCTCTGTGCGATGCGGACATTTACAGCGACTACGAGCAC 165
BP TabermanL1 GCCGAG-TACC--AAATC-AG-CTCT-TCG-ATC--GACATACAC--GACTAC--ACAC 145
BPPICU       GCCGAG-TACC--AAATCGAG-CTCTGTGCG-ATGCGGACATTTACAGCGACTAC--ACAC 155
BP TohamaI    GCCGAG-TACC--AAATCGAG-CTCTGTGCG-ATC--GACATACAC--GACTAC--ACAC 147
                *****

BP18323      GAGCATGGCATCGTGATGACGCCCGATGGCAAGGACGACTACATCAGTTACAGGTCCGCC 219
BP TabermanI  GAGCATGGCATCGTGATGACGCCCGATGGCAAGGACGACTACATCAGTTACAGGTCCGCC 219
BP338BrkA-   GAGCATGGCATCGTGATGACGCCCGATGGCAAGGACGACTACATCAGTTACAGGTCCGCC 240
BP338        GAGCATGGCATCGTGATGACGCCCGATGGCAAGGACGACTACATCAGTTACAGGTCCGCC 225
BP TabermanL1 -AGCATGGCATCGTGATGACGCCCGATGGCAAGGACGACTACATCAGTTACAGGTCCGCC 204
BPPICU       -A-CATGGCATCGTGATGACGCCCGATGGCAAGGACGACTACATCAGTTACAGGTCCGCC 213
BP TohamaI    GAGCATGGCATCGTGATGACGCCCGATGGCAAGGACGACTACATCAGTTACAGGTCCGCC 207
                *****

BP18323      GAGAGCGGTCGTCCGAAGCCCCCCCCCCCCCTTTTCAAACCTTCAAACCATTTGGGTAAT 279
BP TabermanI  GAGAGCGGTCGTCCGAAGCCCCCCCCCCCCCTTTTCAAACCTTCAAACCATTTGGGTAAT 276
BP338BrkA-   GAGAGCGGTCGTCCGAAGCCCCCCCCCCCCCTTTTCAAACCTTCAAACCATTTGGGTAAT 297
BP338        GAGAGCGGTCGTCCGAAGCCCCCCCCCCCCCTTTTCAAACCTTCAAACCATTTGGGTAAT 282
BP TabermanL1 GAGAGCGGTCGTCCGAAGCCCCCCCCCCCCCTTTTCAAACCTTCAAACCATTTGGGTAAT 264
BPPICU       GAGAGCGGTCGTCCGAAGCCCCCCCCCCCCCTTTTCAA-CTTCAAACCATTTGGGTAAT 270
BP TohamaI    GAGAGCGGTCGTCCGAAGCCCCCCCCCCCCCTTTTCAAACCTTCAAACCATTTGGGTAAT 264
                *****

BP18323      GACGTCGTAGCGGAGCGGATACGGGTAGAAGTGCACGGTGACGAAACCCTCGGCGTGTAT 339
BP TabermanI  GACGTCGTAGCGGAGCGGATACGGGTAGAAGTGCACGGTGACTGAACCCTCGGCGTGTAT 336
BP338BrkA-   GACGTCGTAGCGGAGCGGATACGGGTAGAAGTGCACGGTGACGAAACCCTCGGCGTGTAT 357
BP338        GACGTCGTAGCGGAGCGGATACGGGTAGAAGTGCACGGTGACGAAACCCTCGGCGTGTAT 342
BP TabermanL1 GACGTCGTAGCGGAGCGGATACGGGTAGAAGTGCACGGTGACGAAACCCTCGGCGTGTAT 324
BPPICU       GACGTCGTAGCGGAGCGGATACGGGTAGAAGTGCACGGTGACGAAACCCTCGGCGTGTAT 330
BP TohamaI    GACGTCGTAGCGGAGCGGATACGGGTAGAAGTGCACGGTGACGAAACCCTCGGCGTGTAT 324
                *****

BP18323      GTC----- 342
BP TabermanI  GTCGACTCGGAGCACCGTT--- 355
BP338BrkA-   GTCGACTCGGAGCACCGTTTCGC 379
BP338        GTCGACTCGGAGCACCGTTTCGC 364
BP TabermanL1 GTCGACTCGGAGCACCGTTTCGC 346
BPPICU       GTCGACTCGGAGCACCGTTTC- 351
BP TohamaI    GTCGACTCGGAGCACCG----- 341
                ***

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Grey fragments highlighted are assumed to be in-frame shifts, and yellow highlighted regions are poly(C) tracts [HPT]. TGA, red-bold type, is a stop codon.

Figure 3.60 CLUSTAL W (1.83) multiple sequence alignment of 5'-end of *bapC* gene encompassing poly(G) sites

```

BP18323      ---GTGCGTGACAGCACGATAGATGCATACGGCAAGCCGCCTTCCGTCGACTCTCCCGAT 57
BP338BrkA-   -----CGTGACAGCACGATAGATGCATACGGCAAGCCGCCTTCCGTCGACTCTCCCGAT 54
BPPICU       ACGGTGCGTGACAGCACGATAGATGCATACGGCAAGCCGCCTTCCGTCGACTCTCCCGAT 60
BP TabermanI ACCGTGCGTGACAGCACGATAGATGTATACGGCAAGCCGCCTTCCGTCGACTCTCCCGAT 60
BP Tohamai   -----GCATACGGCAAGCCGCCTTCCGTCGACTCTCCCGAT 36
BP TabermanL1 ACGGTGCGTGACAGCACGATAGATGCATACGGCAAGCCGCCTTCCGTCGACTCTCCCGAT 60
BP338        ---GTGCGTGACAGCACGATAGATGCATACGGCAAGCCGCCTTCCGTCGACTCTCCCGAT 57
                * * * * *

BP18323      TATTACGGTGCCGCGGCTGTCTACGCCGGTACGCTGAATATCGAGAATTCCACGGTTTAC 117
BP338BrkA-   TATTACGGTGCCGCGGCTGTCTACGCCGGTACGCTGAATATCGAGAATTCCACGGTTTAC 114
BPPICU       TATTACGGTGCCGCGGCTGTCTACGCCGGTACGCTGAATATCGAGAATTCCACGGTTTAC 120
BP TabermanI TATTACGGTGCCGCGGCTGTCTACGCCGGTACGCTGAATATCGAGAATTCCACGGTTTAC 120
BP Tohamai   TATTACGGTGCCGCGGCTGTCTACGCCGGTACGCTGAATATCGAGAATTCCACGGTTTAC 96
BP TabermanL1 TATTACGGTGCCGCGGCTGTCTACGCCGGTACGCTGAATATCGAGAATTCCACGGTTTAC 120
BP338        TATTACGGTGCCGCGGCTGTCTACGCCGGTACGCTGAATATCGAGAATTCCACGGTTTAC 117
                * * * * *

BP18323      CATAACTATGCGGCCAGCCGTTTCGAAGACGCGGTAGGAGTCGGGGTAACCTCGCTCGGG 177
BP338BrkA-   CATAACTATGCGGCCAGCCGTTTCGAAGACGCGGTAGGAGTCGGGGTAACCTCGCTCGGG 174
BPPICU       CATAACTATGCGGCCAGCCGTTTCGAAGACGCGGTAGGAGTCGGGGTAACCTCGCTCGGG 180
BP TabermanI CATAACTATGCGGCCAGCCGTTTCGAAGACGCGGTAGGAGTCGGGGTAACCTCGCTCGGG 180
BP Tohamai   CATAACTATGCGGCCAGCCGTTTCGAAGACGCGGTAGGAGTCGGGGTAACCTCGCTCGGG 156
BP TabermanL1 CATAACTATGCGGCCAGCCGTTTCGAAGACGCGGTAGGAGTCGGGGTAACCTCGCTCGGG 180
BP338        CATAACTATGCGGCCAGCCGTTTCGAAGACGCGGTAGGAGTCGGGGTAACCTCGCTCGGG 177
                * * * * *

BP18323      GATAAGGCCATACTCAACGTTACCGACAGCGAGGTATCGGGTGCGAGGGGCGCGGTCATC 237
BP338BrkA-   GATAAGGCCATACTCAACGTTACCGACAGCGAGGTATCGGGTGCGAGGGGCGCGGTCATC 234
BPPICU       GATAAGGCCATACTCAACGTTACCGACAGCGAGGTATCGGGTGCGAGGGGCGCGGTCATC 240
BP TabermanI GATAAGGCCATACTCAACGTTACCGACAGCGAGGTATCGGGTGCGAGGGGCGCGGTCATC 240
BP Tohamai   GATAAGGCCATACTCAACGTTACCGACAGCGAGGTATCGGGTGCGAGGGGCGCGGTCATC 216
BP TabermanL1 GATAAGGCCATACTCAACGTTACCGACAGCGAGGTATCGGGTGCGAGGGGCGCGGTCATC 240
BP338        GATAAGGCCATACTCAACGTTACCGACAGCGAGGTATCGGGTGCGAGGGGCGCGGTCATC 237
                * * * * *

BP18323      GGTGTTGGGGGGGGG-----AAGCGACATTT-ACCGATTCGGTCCTGCGT-GGTTTCGGCCT 289
BP338BrkA-   GGTGTTGGGGGGGGGGGGG---AAGCGACATTT-ACCGATTCGGTCCTGCGT-GGTTTCGGCCT 289
BPPICU       GGTGTTGGGGGGGGGGGGG--GAAGCGACATTTTACCGATTCGGTCCTGCGTTGGTTTCGGCCT 298
BP TabermanI GGTGTTGGGGGGGGGGGGG--GAA-CGACATTT-ACCGATTCGGTCCTGCGT-GGTTTCGGCCT 295
BP Tohamai   GGTGTTGGGGGGGGGGGGCAAGAAAGCGACATTT-ACCGATTCGGTCCTGCGT-GGTTTCGGCCT 274
BP TabermanL1 GGTGTTGGGGGGGGGGGG---GAAGCGACATTT-ACCGATTCGGTCCTGCGT-GGTTTCGGCCT 295
BP338        GGTGTTGGGGGGGGGGGG---GAAGCGACATTT-ACCGATTCGGTCCTGCGT-GGTTTCGGCCT 292
                * * * * *

BP18323      TCGGGCTGTACGCCGAAATGTGCGACACCTGCAGAGATGATGATGGCACCTCGCCTTCGA 349
BP338BrkA-   TCGGGCTGTACGCCGAAATGTGCGACACCTGCAGAGATGATGATGGCACCTCGCCTTCGA 349
BPPICU       TCGGGCTGTACGCCGAAATGTGCGACACCTGCAGAGATGATGATGGCACCTCGCCTTCGA 358
BP TabermanI TCGGGCTGTACGCCGAAATGTGCGACACCTGCAGAGATGATGATGGCACCTCGCCTTCGA 355
BP Tohamai   TCGGGCTGTACGCCGAAATGTGCGACACCTGCAGAGATGATGATGGCACCTCGCCTTCGA 334
BP TabermanL1 TCGGGCTGTACGCCGAAATGTGCGACACCTGCAGAGATGATGATGGCACCTCGCCTTCGA 355
BP338        TCGGGCTGTACGCCGAAATGTGCGACACCTGCAGAGATGATGATGGCACCTCGCCTTCGA 352
                * * * * *

BP18323      TTCGCGTCCAAGGCGGGGTTGTTTACGGGCGGCATGGGTGCAAAATAACGCTC----- 409
BP338BrkA-   TTCGCGTCCAAGGCGGGGTTGTTTACGGGCGGCATGGGTGCAAAATAACGCTC----- 400
BPPICU       TTCGCGTCCAAGGCGGGGTTGTTTACGGGCGGCATGGGTGCAAAATAACG----- 406
BP TabermanI TTCGCGTCCAAGGCGGGGTTGTTTACGGGCGGCATGGGTGCAAAATAACGTC----- 405
BP Tohamai   TTCGCGTCCAAGGCGGGGTTGTTTACGGGCGGCATGGGTGCAAA----- 378
BP TabermanL1 TTCGCGTCCAAGGCGGGGTTGTTTACGGGCGGCATGGGTGCAAAATAACGTC----- 405
BP338        TTCGCGTCCAAGGCGGGGTTGTTTACGGGCGGCATGGGTGCAAAATAACGTCCT----- 404
                * * * * *

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Yellow highlighted regions are poly(G) tracts [HPT].

Table 3.4 Number of C and G residues in the *bapC* poly(C) and poly(G) tracts in *B. pertussis* strains

DNA source	Type of DNA sequenced	No. of C residues in poly(C) tract	No. of G residues in poly(G) tract
BP18323	Amplicon	15	9
BP PICU	Amplicon	13	11
BP Taberman (L1)	Amplicon	14	12
BP Taberman (I)	Amplicon	13	11
BP338	Amplicon	13	12
BP338 (BrkA ⁻)	Amplicon	13	12
BP Tohama I (Glasgow strain)	Amplicon	13	11
BP Tohama (Actual genome sequence, with frame shift)	Chromosomal library clone	14	11
BP Tohama published sequence without frameshift	Chromosomal library clone	13	9

Downstream of the poly(C) tract in *B. pertussis* BP Taberman I there was, surprisingly, a stop codon (TGA, red-bold type), which could be a true or false termination as a consequence of PCR reaction.

Interestingly, the region 5'-upstream of the poly(C) fragment in BP Tohamal strain, BP Taberman (L1) and BP PICU showed an in-frame shift of a fragment containing 40-47 nucleotides (Fig. 3.59, grey highlighted fragments).

Analysis of the segment encompassing the homopolymeric tract of 9 guanine residues in *B. pertussis* strains exhibited a polymorphism of 9-12 (G) nucleotides (Fig. 3.60). *B. pertussis* strains such as BP Taberman (I), BP PICU and BP Tohama (Glasgow strain) showed no difference in the number of (G) nucleotides compared to the BP Tohama genome sequence, published in the database. However, *B. pertussis* strains BP Taberman (L1), BP338 and BP338 (BrkA⁻) had a G (12) tract with an extra (G) nucleotide compared to those described above. Table 3.4 shows the number of C and G nucleotides in the HPT variable segments of *bapC* gene.

3.21.2 Amino acid sequence analysis of *bapC* gene region spanning the poly(C) and poly(G) tracts of different *B. pertussis* strains

In view of the above variations in the homopolymeric tracts and flanking regions of the *bapC* gene in different *B. pertussis* strains, it was important to determine whether such changes would allow expression of the full-length BapC protein. As explained above, the frameshifts in the actual sequence of the *B. pertussis* Tohama genome strain would not allow this.

Figure 3.61 CLUSTAL W (1.83) multiple sequence alignment of amino acids at N-terminus of BapC, corresponding to region containing the poly(C) sequences

```

BP18323      -----AARYLRFQVAVAGVAAGALPSSDVDAQAAPAAAEVAKIEALSDADIYSDYEH 53
BP338BrkA-   KSNMSPAARYLRFQVAVAGVAAGALPSSDVDAQAAPAAAEVAKIEALSDADIYSDYEH 60
BP TabermanI -----AARYLRFQVAVAGVAAGALPSSDVDAQAAPAAAEVAKIEALSDADIYSDYEH 53
BP338        -----SPAARYLRFQVAVAGVAAGALPSSDVDAQAAPAAAEVAKIEALSDADIYSDYEH 55
BP Tohamai    -----AARYLRFQVAVAGCAAGALPSSDVDAQAAPAAAEY-QIELCRS-TYTTT--H 49
BPPICU        -----PAARYLRFQVAVAGVAAGALPSSDVDAQAAPAAAEY-QIELCRSGHLQRL--H 51
BP TabermanL1 -----AARYLRFQVAVAGVAAGALPSSDVDAQAAPAAAEY-QIS--SSIDIHDY--T 48
               *****
               *****

BP18323      EHGIVMTPDGKDDYISYRSAESGRPKPPPPF-SNFKPLGNDVVAERIRVEVHGDETLGVY 113
BP338BrkA-   EHGIVMTPDGKDDYISYRSAESGRPKPPPP-LSNFKPLGNDVVAERIRVEVHGDETLGVY 119
BP TabermanI EHGIVMTPDGKDDYISYRSAESGRPKPPPP-LSNFKPLGNDVVAERIRVEVHGDETLGVY 111
BP338        EHGIVMTPDGKDDYISYRSAESGRPKPPPP-LSNFKPLGNDVVAERIRVEVHGDETLGVY 114
BP Tohamai    EHGIVMTPDGKDDYISYRSAESGRPK-PPPLSNFKPLGNDVVAERIRVEVHGDETLGVY 108
BPPICU        THGIVMTPDGKDDYISYRSAESGRPK-PPPLSNFKPLGNDVVAERIRVEVHGDETLGVY 110
BP TabermanL1 QHGIVMTPDGKDDYISYRSAESGRPKAPPPF-SNFKPLGNDVVAERIRVEVHGDETLGVY 108
               *****
               *****

BP18323      V----- 114
BP338BrkA-   VDSEHRS 126
BP TabermanI VDSEHR- 117
BP338        VDSEHRS 121
BP Tohamai    VDSEH-- 113
BPPICU        VDSEHRS 117
BP TabermanL1 VDSEHRS 115
               *

```

The yellow highlighted fragments are in-frame shifts upstream to poly amino acid proline, grey regions.

Figure 3.62 CLUSTAL W (1.83) multiple sequence alignment of amino acids at N-terminus of BapC, corresponding to region containing the poly(G) sequences

```

BP Tohamai    -----AYGKPPSVDSPDYGGAAAVYAGTLNIENSTVHHNYAAQPFEDAVGVGVTSLG 52
BP18323      -VRDSTIDAYGKPPSVDSPDYGGAAAVYAGTLNIENSTVHHNYAAQPFEDAVGVGVTSLG 59
BP338BrkA-   --RDSTIDAYGKPPSVDSPDYGGAAAVYAGTLNIENSTVHHNYAAQPFEDAVGVGVTSLG 58
BP338        -VRDSTIDAYGKPPSVDSPDYGGAAAVYAGTLNIENSTVHHNYAAQPFEDAVGVGVTSLG 59
BP TabermanL1 TVRDSTIDAYGKPPSVDSPDYGGAAAVYAGTLNIENSTVHHNYAAQPFEDAVGVGVTSLG 60
BP TabermanI TVRDSTIDVYGKPPSVDSPDYGGAAAVYAGTLNIENSTVHHNYAAQPFEDAVGVGVTSLG 60
BPPICU        TVRDSTIDAYGKPPSVDSPDYGGAAAVYAGTLNIENSTVHHNYAAQPFEDAVGVGVTSLG 60
               .*****

BP Tohamai    DKAILNVTDSEVSGARGAVIGWGGGQ-EATFTDSVLRGSAGFLYAEMCDTCRDDGTSPSI 112
BP18323      DKAILNVTDSEVSGARGAVIGWGG--EATFTDSVLRGSAGFLYAEMCDTCRDDGTSPSI 117
BP338BrkA-   DKAILNVTDSEVSGARGAVIGWGGG-EATFTDSVLRGSAGFLYAEMCDTCRDDGTSPSI 117
BP338        DKAILNVTDSEVSGARGAVIGWGGG-EATFTDSVLRGSAGFLYAEMCDTCRDDGTSPSI 118
BP TabermanL1 DKAILNVTDSEVSGARGAVIGWGGG-EATFTDSVLRGSAGFLYAEMCDTCRDDGTSPSI 119
BP TabermanI DKAILNVTDSEVSGARGAVIGWGGG-RTTFTDSVLRGSAGFLYAEMCDTCRDDGTSPSI 119
BPPICU        DKAILNVTDSEVSGARGAVIGWGGGRSDILPIFSCVGSAGFLYAEMCDTCRDDGTSPSI 120
               *****
               *****

BP Tohamai    RVQGGVVQGGMGAN----- 126
BP18323      RVQGGVVQGGMGANNAEMCDTCRDDGTSPSIRVQGGVVQGGMGANN 164
BP338BrkA-   RVQGGVVQGGMGANNV----- 133
BP338        RVQGGVVQGGMGANNV----- 134
BP TabermanL1 RVQGGVVQGGMGANNV----- 135
BP TabermanI RVQGGVVQGGMGANNV----- 135
BPPICU        RVQGGVVQGGMGANN----- 135
               *****

```

The yellow highlighted fragment is an in-frame shift downstream to poly amino acid glycine, grey regions.

Translated and deduced amino acid sequences (Figs 3.61 and 3.62) using ExPASy translate tool (<http://www.expasy.org/tools/dna.html>) were analyzed for the different *B. pertussis* strains and multiple alignments of the amino acid sequences were created with the CLUSTALW program.

The translated region spanning the poly(C) tract in *B. pertussis* strains (Fig. 3.61, grey highlighted fragments) showed no difference in the number of proline residues (4), except for BP18323 (5), compared to the BP Tohama (genome) amino acid sequence published in the database. An extra in-frame amino acid proline and leucine replacement by phenylalanine were observed in BP18323 strain due to insertion of extra two (C) nucleotides. In addition, the region 5'-upstream of the poly(C) fragment in BP TohamaI strain, BP Taberman (L1) and BP PICU showed an in-frame shift of 12-15 amino acids (Fig. 3.61, yellow highlighted regions). No hit was found using ScanProsite (<http://www.expasy.org/tools/scanprosite>) release 20.11 tool to retrieve any identical or similar pattern, profile or motif stored in the database, in comparison with this variable 15 amino acids segment. Nevertheless, a possible function was obtained for the conserved fragment (VAKIEALSDADIYSD) when compared with motifs stored in the Protein Blast database (www.ncbi.nlm.nih.gov/blast) (BLASTP 2.2.16 release). This motif was a part of periplasmic binding protein (PBP) using for transporting a wide variety of substrates, such as amino acids, peptidases, sugars, vitamins and inorganic ions via bacterial periplasmic transport systems and may indicate an evolutionary or environmental compatibility at this region. Taking account of Cs and flanking regions, the sequences of the most of strains were in-frame and would not prevent them expressing BapC.

Analysis of translated and deduced amino acid sequences using ExPASy tool and multiple alignment of the amino acid sequences of the poly(G) tracts showed an extra in-frame glycine codon in all of the *B. pertussis* strains, except for BP18323, when compared with the Tohama genome sequence in the database (Fig. 3.62). Moreover, the region 3'-downstream

of the poly(G) tract in BP PICU strain showed an in-frame shift of 11 amino acids (Fig. 3.62, yellow highlighted fragment). However, no hit was again found using ScanProsite (<http://www.expasy.org/tools/scanprosite>) release 20.11 tool to retrieve the occurrence of any identical or similar pattern, profile or motif stored in the database in comparison with this variable 11-amino acid segment (RSDILPIRSCV), although it showed a resemblance to a periplasmic binding protein using for transporting a wide variety of substrates by bacterial periplasmic transport systems, using Protein BLAST search (www.ncbi.nlm.nih.gov/blast) (BLASTP 2.2.16 release). Moreover, an additional search using BLASTp for the 11-amino acid conserved fragment (GEATFTDSVLR) in the other *B. pertussis* strains compared in this study showed an 88% identity with a hypothetical protein of *Collinsella aerofaciens*.

It can therefore be concluded from the above observations that, although the entire *bapC* gene was not sequenced in these different *B. pertussis* strains, there are some minor nucleotide and amino acid changes in some strains but it is likely that all would be able to produce some form of BapC, unlike the *B. pertussis* Tohama genome strain. Thus, the data supported the findings by Blackburn (2000) and Bokhari (2002) that *B. pertussis* Tohama (Glasgow strain) and Taberman produce BapC protein.

4. Discussion

4.1 Overview

The autotransporters are a family of extracellular proteins found in many Gram-negative bacteria. Several of these proteins are known to play essential roles in the pathogenesis of bacterial infections and have been characterised in detail. However, most of the autotransporter proteins remain to be characterised. The main three characteristics of these proteins are that most of the mature proteins are processed into a large N-terminal domain and an approximately 30-kDa C-terminal domain, the C-terminal domain is predicted to produce an amphipathic β -barrel in the outer membrane, creating an integral outer-membrane pore and, finally, export of these proteins through the outer membrane does not necessitate accessory proteins, as their names imply.

The *bordetellae* have the capacity to encode 22 of these proteins (Table 1.1), of which a few (BrkA, pertactin, SphB1, TcfA and Vag8) have been assigned functions in host interaction and virulence (Parkhill *et al.*, 2003). However, *B. pertussis* (13) and *B. parapertussis* (16) each have fewer autotransporter ORFs and more autotransporter pseudogenes compared with *B. bronchiseptica* (20), although it should be noted that *bapC* was not shown as a pseudogene by Parkhill *et al.* (2003). The number of autotransporter pseudogenes is reflected in the overall genome structure, where *B. bronchiseptica* has only 18 (0.4% of the total number of genes) pseudogenes, whereas *B. pertussis* and *B. parapertussis* have 358 (9.4%) and 220 (5%), respectively (Preston *et al.*, 2004). The greater number of autotransporters and surface-protein coding capacity of *B. bronchiseptica* could be a reflection of its abilities to bind to tissues in, and infect, a wider range of hosts.

One of the *Bordetella* autotransporter genes (initially named *bap-5*; Appendix II.3) was first identified due to a misprimed PCR reaction designed to amplify the *prn* gene from the

B. pertussis genome (Blackburn, 1998). An identical gene/protein was later identified and named BapC, the product of *bapC* gene, by Henderson *et al.* (2000), and this terminology has been adopted in the present study. A database search showed that homologous genes were also present in *B. parapertussis* and *B. bronchiseptica* (Blackburn, 2000). The structural similarities between BapC (the product of *bapC*) and other, already well-characterised *B. pertussis* autotransporter proteins i.e. PRN, TCF and BrkA (Fig. 1.11) suggested that the BapC protein might have similar functions, perhaps as an adhesin or a serum resistance factor. Similarities such as the integrin-binding motif (RGD), glycosaminoglycan-binding site (SGXG), proteolytic processing site (ALSKRLGEL) and outer membrane localisation signal, which ends at a cyclic amino acid like other autotransporters in Gram-negative bacteria (FHLGYRTW), are common outstanding features of autotransporters.

Further work by Bokhari (2002) had provided preliminary evidence that BapC was a putative virulence factor of *B. pertussis*, providing resistance to serum killing, by interfering with the classical pathway of complement activity (like BrkA), and possibly adherence to certain mammalian cell types. The aim of the present project was to further characterize this potentially important component. However, during the course of this thesis, further interesting, and apparently conflicting data on BapC were reported. With the publication of the genome sequences of three *Bordetella* strains (Parkhill *et al.*, 2003), it became evident that the sequences reported by Blackburn (1998) and Henderson (2000) did not reflect the complete gene or protein. Although the three *Bordetella* species were reported as containing a number of autotransporter pseudogenes, *bapC* was not shown as a pseudogene in any of the species (Parkhill *et al.*, 2003). The subsequent literature on BapC has also been confusing. For example, in the table of Henderson *et al.*, (2004) (Table 1.1) BapC is shown as protein of 998 amino acids in *B. bronchiseptica* but in *B. pertussis* Tohama and *B. parapertussis* as 102 and 100 amino acids, truncated due to frameshift or insertion of a stop codon. GenBank data by Sebaihia in 2006 reported BapC as putative

autotransporter with 993 amino acids ORF in *B. bronchiseptica* (Accession No. NP_888576) but as a pseudogene in *B. pertussis* (Accession No. NP_881344) and *B. parapertussis* (Accession No. NP_884815). To add to the confusion, the amino acid sequence of BapC in *B. pertussis* and *B. parapertussis* in GenBank and the corresponding nucleotide sequences of *B. pertussis* and *B. parapertussis* in GenBank show full-length proteins and the unmutated genes, respectively.

In previous studies from our laboratory, by RT-PCR and the immunoblotting with a rabbit anti-BapC serum (as used in the present study), it appeared that the *bapC* gene was expressed in *B. pertussis* strains Tohama and Taberman (Bokhari, 2002). The RT-PCR results also suggested that *bapC* expression was regulated by the Bvg two-component regulatory system. Moreover, disruption of the *bapC* gene in these two strains of *B. pertussis* gave mutants with altered properties such as increased serum sensitivity, reduced adhesion to the non-respiratory HeLa cells and to human respiratory epithelial laryngeal (Hep-2) cells and reduced virulence for mice by the intranasal route (Bokhari, 2002). In view of these apparent discrepancies, further genetic and functional characterisation of BapC was undertaken in the present study.

4.2 Construction of *bapC*-defective mutants

Although, *bapC* mutants were available from previous work, the rational for creating BP338 (BapC⁻) and BP338 (BrkA⁻, BapC⁻) mutants was to determine the exact role of BapC in the same genetic background.

The *bapC*-defective mutants of *B. pertussis* were created using the suicide vector pSS1129 (Stibitz, 1986) for gene replacement. This vector had been used previously for creating mutants in *B. pertussis* e.g. *fhaB*-defective and *bvgA*-defective mutants. The potential usefulness of this specific vector in different bacterial species other than *E. coli* depends on the proper function of two basic aspects, its capacity for transfer to, but inability to

replicate in these species. The construct has an origin of transfer from plasmid RP4 and, provided that *tra* functions are supplied *in trans*, the vector will be transferred into the recipient bacterium by conjugation. The ligation of the mutated (with inserted tetracycline resistance cassette) *bapC* gene into the pSS1129 vector proved to be a difficult task, probably due to the high GC-content of the *bapC* gene. The derived streptomycin resistant strain of *B. pertussis* provided a valuable selection marker for selecting the exconjugant strains of *B. pertussis* and also for inhibiting the growth of the undesired DNA-mobilising *E. coli* strain SM10.λpir.

The rationale behind the two-step selection used is that the first cross-over is accomplished by selecting for the vector or a marker within the cloned sequence, since the vector is a member of ColE1 family and is not able to replicate in *B. pertussis* and many other bacteria. The first step was therefore to select for those bacteria in which the plasmid had integrated into the chromosome. If the cloned segment within the plasmid was homologous to the chromosome, integration would very likely occur via homologous recombination. The second step was used to select for the loss of the integrated plasmid. This was accomplished because of the wild-type *E. coli* *rspL* gene in pRTP1 (pSS1129) that confers a streptomycin-sensitive phenotype on strains. Streptomycin-resistant survivors would therefore be those which had lost the plasmid copy of *rpsL*. This loss occurs by the mechanism of homologous recombination between the direct repeats, which flank the vector as a result of its integration. As a result of this manipulation, two crossovers would have occurred between the plasmid with the encoded gene and chromosomal copies of the gene in question. If these crossovers occurred on opposite sides of the site of alteration in the cloned gene, the net result is that the desired alteration would be obtained. If the crossovers occur at the same side, the original allele would remain (Fig. 3.30). PCR and Southern blotting results showed the successful incorporation of the mutated *bapC* into the chromosome of *B. pertussis* strain 338 wild type and its *brkA* mutant (BPM2041).

Some unwanted background reactions were found at each recombination step. For example, at the first crossover when there is no cloned homologous segment present in the vector, one can still isolate at a low frequency, insertions of the vector into the chromosome. These illegitimate insertions do not occur via homologous recombination as a result of sequence similarity between plasmid and chromosomal homology of genes as they can occur at random sites. Another background reaction may appear at the second crossover. If survivors are selected for the second plasmid marker such as ampicillin resistance, some of them contain this marker and some do not. It has been suggested that they may have been obtained via some undefined or less defined mechanisms involving spontaneous deletion. These exceptions occur when attempting to do allelic exchange under non- or less optimal conditions. If there is a sufficient flanking homology, for example 500 bp on each side of the mutation, and if a reasonable screen exists for incorporation of the mutation, this background will happen with only a low frequency.

Two more points have to be borne in mind when using mutant strains. As the mutagenesis is performed by inserting a cassette downstream of the predicted translational start site, there could be the possibility of getting truncated peptides and that could cause difficulties in defining the role of the gene. Another possibility is the effect of the mutagenesis on neighbouring genes (polar effect). In the case of the *bapC* gene, a database search of the *bapC* flanking regions using the *B. pertussis* Tohama I genome sequence (BLAST search at http://www.Sanger.ac.uk/projects/B_pertussis) contig BX640419 indicated the presence of a probable ammonium transporter gene (*amtB*, locus_tag BP2737) 5'-upstream of *bapC* and a hypothetical gene encoding a putative TetR-family transcriptional regulator (locus_tag BP2741) located 3'-downstream of the *bapC* gene. These two loci do not therefore appear to form a part of an operon with *bapC* and mutagenesis of *bapC* would be unlikely to have any effect on these neighbouring genes. In fact, by immunoblotting, the amount of BrkA in BapC mutants (Figs 3.2 and 3.3) appeared to be somewhat reduced, as if *bapC* mutation had some effect on BrkA expression. However, the *brkA* gene (locus_tag

3494) is far away from *bapC* gene (locus_tag 2738) on the *B. pertussis* genome and it is unlikely that mutation in that *bapC* gene could have any effect on *brkA* gene and also there is no evidence that *brkA* and *bapC* genes are regulated in an operon, where any effect on one transcript may affect the following transcripts. One possible line of future work would be to restore BapC expression to the BapC-deficient mutants by returning the *bapC* gene on a plasmid, to investigate the effect of BapC on BrkA expression.

4.3 Role of BapC in serum resistance

A range of surface-associated or released virulence factors is employed by *B. pertussis* to survive in its host, and to delay or evade the immune effector mechanisms deployed against it. *B. pertussis* is a mucosal pathogen; nevertheless it can be exposed to levels of complement about 20% of that of normal human serum due to extravasation or plasma exudation via valve-like openings between epithelial cells (Persson, 1991). However, the mucosal exudation of plasma characteristically occurs in health and disease; levels may be further increased during inflammation (Persson, 1991; Barnes and Weiss, 2001). This organism, like other mucosal pathogens appears to possess mechanisms in place to resist complement (Weiss *et al.*, 1999).

However, *B. pertussis* does show some sensitivity to complement killing and this is attributed in part to the absence of O-side chain on its LOS. *B. bronchiseptica* and *B. parapertussis* strains with the longer LPS and expressing of O antigens are completely resistant to naïve serum complement whereas O-chain defective mutants are very sensitive to complement (Burns *et al.*, 2003). In the absence of O antigens, *B. pertussis* does therefore require other mechanisms to resist complement.

It had been suggested earlier that BapC has a role in *B. pertussis* Taberman and Tohama in conferring some resistance to complement-mediated killing. BapC was shown to confer a resistance on *B. pertussis* Taberman and Tohama to killing by normal human serum

compared to their BapC-deficient mutants (Bokhari, 2002). Moreover, it was observed that BapC protects *B. pertussis* mainly from the classical (antibody-dependent) pathway of complement activation (Bokhari, 2002). The present study has also confirmed that BapC may play a role in protecting *B. pertussis* from killing by normal human serum since the BapC mutant strains: BP Taberman (BapC⁻), BP Tohama (BapC⁻) (section 3.6), BP338 (BapC⁻) and BP338 (BrkA⁻, BapC⁻) (section 3.15) were more sensitive than their parents to killing by normal human serum. BapC was shown to confer a considerable resistance on *B. pertussis* BP338 to killing by normal human serum compared to its BapC-deficient mutant ($P<0.05$). Moreover, it was demonstrated that the BP338 (BrkA⁻, BapC⁻) double mutant was significantly ($P<0.05$) more sensitive to killing by complement than the single BrkA⁻ or BapC⁻ mutants in the same genetic background (BP338). It was also shown that the killing of *B. pertussis* BP338 and its BrkA⁻ and BapC⁻ single and BrkA⁻, BapC⁻ double mutants, grown under modulating conditions, was significantly ($P<0.05$) greater when exposed to normal human serum compared to those grown under non-modulating conditions. Moreover, although the double mutant was more serum sensitive than either the BrkA⁻ or BapC⁻ single mutants, it was not as sensitive as the Bvg⁻ derivative of BP338 strain (BP347). These data suggest that although BapC, in addition to BrkA, seems to play a role in conferring resistance on *B. pertussis* to killing by complement, other as yet unidentified *bvg*-regulated factors may also be involved in serum resistance.

The complement system is part of the innate immune defences, and provides a first line of defence against pathogens that the host has not encountered previously. The complement system can be activated in several ways. The classical pathway is usually activated by deposition of antibody on the bacterial surface via binding of C1 protein to the Fc regions of two antibodies in close proximity, which changes the C1 conformation so that it becomes proteolytically active. However, in the absence of antibody, C1 can directly activate the classical pathway by binding to bacterial targets such as porins (Alberti *et al.*, 1995; Barnes and Weiss, 2003). Complement is also activated by binding of mannose-

binding-lectin (MBL) to mannose residues on the bacterial surface which activates C4 and C2 of the classical pathway in a manner similar to the antibody/C1 activation process. This lectin pathway is an antibody-independent route of the classical pathway. MBL has structurally similar to C1q and is able to activate complement via associated serine proteases (MASP-1 and MASP-2) (Matsushita and Fujita, 1992). Killing is mediated by the terminal lytic complement pathway or possibly by increasing phagocytosis. However, a role for the MBL pathway has been ruled out in killing of *B. pertussis* with intact LPS (LOS) since mannose is not a component of the *B. pertussis* LPS (LOS) (Barnes and Weiss, 2003).

The alternative pathway, the second complement pathway, is activated by carbohydrates on the surface of bacteria (such as LPS). However, the surface of *B. pertussis* appears not to be able to activate the alternative pathway of complement (Fernandez and Weiss, 1994; Barnes and Weiss, 2003) and *B. pertussis* is killed in the absence of the alternative pathway suggesting that only the classical pathway of complement mediates killing of *B. pertussis* (Barnes and Weiss, 2003). Furthermore, it has been suggested that resistance to the alternative pathway in some bacterial pathogens is achieved due to modification of their LPS by addition of sialic acid in particular (Parsons *et al.*, 1989). There is no report that *B. pertussis* can modify its LPS (LOS) in this way.

It has been shown that naïve serum resistance is acquired by both BrkA and Bvg mutants of *B. pertussis* *in vivo* (Pishko *et al.*, 2003). A BrkA mutant of BP338 (RFBP 2152) acquired a significant amount of resistance *in vivo*, but the level of resistance acquisition was not as high as in wild-type strains which suggested that the BrkA protein is not involved in the acquisition of resistance to complement (alternative pathway) killing *in vivo* (Pishko *et al.*, 2003). This *in vivo* acquisition of complement resistance suggested that there could be a novel O antigen-independent method by which *B. pertussis* evades complement-mediated killing (Pishko *et al.*, 2003). It is therefore possible that *B. pertussis*

has more than one mechanism for complement evasion (Berggard *et al.*, 2001) and the BrkA-dependent mechanism may be more effective against the antibody-mediated classical pathway killing during the adaptive immune response. However, it has been shown that the BrkA is involved in resisting both antibody-independent and –dependent pathways of complement killing *in vitro* (Elder and Harwill, 2004) and increased sensitivity of all *brkA* mutants in *B. pertussis* isolates to both naïve and immune serum suggested a noncritical or redundant function for BrkA in complement resistance to killing (Elder and Harwill, 2004). It was also found that the critical function of BrkA *in vivo* was independent from its effects in resisting to lysis mediated by complement, and there was no correlation between *in vitro* sensitivity to complement and *in vivo* infection ability (Elder and Harwill, 2004). This phenomenon might be justified by the fact that *B. pertussis* strains can even acquire resistance to complement-mediated killing during the first 24 h of infection, in the absence of BrkA or other regulated genes by BvgAS system (Pishko *et al.*, 2003). In addition, the high level of acquired complement resistance is specific to *B. pertussis* since wild-type *B. bronchiseptica* and *B. parapertussis* are normally resistant to the alternative complement pathway and it is doubtful whether they acquire resistance to the same extent as *B. pertussis* (Pishko *et al.*, 2003).

It has been shown that the BrkA protein of *B. pertussis* inhibits killing by the antibody-dependent classical pathway of complement via the inhibition of deposition of C4, C3 and C9 and production of soluble membrane attack complex (Barnes and Weiss, 2001). In contrast, BrkA does not affect the deposition of C1, suggesting that BrkA possibly acts before C4 (the second component of the classical pathway to be activated) deposition.

Recently, it has also been reported that bacterial concentrations greater than approximately 10^7 CFU/ml deplete complement, resulting in decreased bacterial killing (Burns *et al.*, 2003) and the limited amount of complement in 10% serum has little effect on bacterial concentrations greater than approximately 10^9 CFU/ml, even with the most sensitive

strains (Burns *et al.*, 2003). In addition to substantial variation in sensitivity between strains, it is possible that various growth conditions contribute to differences in the sensitivities of *B. pertussis* strains to complement (Pishko *et al.*, 2003). Pishko *et al.* (2003) showed that growth on BG plates with blood, in contrast to growth in SS broth or on plates without blood, significantly increased the serum resistance of *B. pertussis* although the mechanism for this is unknown. In the present study, all *B. pertussis* strains were grown under the same conditions, on BG agar then suspended in SS-medium and exposed to normal human serum, at dilutions up to 1/80 at a concentration of 4×10^8 CFU/ml. Up to >99% killing of the most sensitive strains occurred with 45 min and there were clear differences between the sensitive and more resistant (wild-type) strains.

It has also been shown that complement susceptibility of *B. pertussis* is growth stage specific but was not due to differential expression of BrkA, suggesting that factors in addition to BrkA play a role in conferring resistance to complement (Barnes and Weiss, 2002). In the present study, the effect of growth phase on serum sensitivity was not examined, but all *B. pertussis* strains were grown under the same conditions so differences in serum sensitivity would not be due to differences in growth phase.

Since one of the first lines of defence in the innate immunity is typically the complement system, bacteria have developed different ways to circumvent the effects of complement, including coating themselves in complement inhibitors (such as C1q binding protein, factor H, C4BP and so on). Although *B. pertussis* FHA can bind to C4 binding protein (C4BP), it did not appear to have a role in serum resistance (Fernandez and Weiss, 1998; Berggard *et al.*, 2001). Binding of complement inhibitor factor H (Amdahl *et al.*, 2006) and C1 esterase inhibitor (Marr *et al.*, 2007) have been reported recently but the surface components involved have not yet been characterised. The mechanism of BapC action in resistance to complement was not examined in this study and further investigation would be required to determine, for example, whether it can bind to or inhibit key components of the various

complement pathways. The availability of recombinant BapC from *E. coli* pET33b (BapC) (section 3.20), constructed in this study, should greatly facilitate this investigation.

Another line of investigation would be to restore the ability of the BapC-deficient mutants to resist killing by serum or complement by allowing the spontaneous deposition of purified recombinant BapC protein on their surface and then performing serum killing assays on these strains along with parent and untreated *bapC* mutant strains. A similar experiment was performed using a purified preparation of BrkA with a *brkA* mutant and the outcome of the experiment confirmed the role of BrkA in serum resistance (Oliver and Fernandez, 2002). Another investigation would be to restore resistance to serum killing by putting back a wild-type copy of the *bapC* gene on a plasmid into the *bapC*-mutant strains of *B. pertussis*, in order to confirm the phenotype conferred by *bapC*.

4.4 Expression of recombinant BapC

To determine the role of BapC protein in serum resistance, a large portion of the *bapC* gene that had been cloned in pQE60 plasmid by Blackburn (2000) was expressed in *E. coli* after IPTG induction. Although this part of BapC should not have been expressed on the surface of *E. coli* because of the lack of a signal peptide in its sequence, necessary to target the nascent protein to the inner-membrane, the recombinant strain showed a strong agglutination reaction with anti-BapC serum. This suggested the presence of BapC on the surface and, interestingly, it did confer significant (P value <0.05) serum resistance to the host strain when compared to the uninduced strain. This difference was apparent when the strains were exposed to the 1/5 dilution of the normal human serum (NHS), which is approximately the same concentration as in human lungs (Persson, 1991). Thus, the incomplete BapC protein, without the signal sequence, appeared to be reaching the *E. coli* cell surface and conferring serum resistance. However, expression of only part of the N-terminal sequence (NTS) of the BapC protein without the β -domain conferred no

significant resistance in the serum killing assay, at either 1/5 serum dilution of serum or at 1/10 dilution. It was therefore necessary, in the light of *B. pertussis* complete genome sequence published in database, to express the whole BapC protein, with and without the signal sequence, to investigate the role of BapC in serum resistance, or in other virulence functions, away from other factors in *B. pertussis*.

The identification of the BapC protein in the *E. coli* expression strain and the resulting peptide fingerprinting, after manually excising the bands of interest from an SDS-PAGE gel and subjecting the information obtained from MS to the database search, showed that the ~90 kDa band corresponded to the unprocessed form of BapC protein published in the database (locus_tag BP2738) with 46% known-peptide coverage. This clearly showed that the BapC in *B. pertussis* strain BP338, a Tohama derivative, would indeed be expressed in this strain.

The properties of *E. coli* pET33b (BapC \pm SS) strains were not investigated in the present study because of lack of time. However, it would clearly be of interest to compare the serum sensitivity of the parent *E. coli* pET33b without insert, and those strains expressing full-length BapC, with and without the signal sequence. It was surprising that *E. coli* pQE60 (ATG1) expressing the incomplete protein, without the signal sequence and part of the N-terminus appeared to express the protein on its surface, as judged by its strong agglutination by anti-BapC serum and increased resistance to complement. Similar studies could be done with *E. coli* pET33b (BapC) strains, without induction or induced perhaps to different levels with IPTG, so that the protein is not grossly over-expressed. It would be of great interest to determine if the recombinant BapC is exported and processed in the same way as in *B. pertussis*. The great advantage of expressing BapC in *E. coli* is that it would be away from other potential serum resistance factors of *B. pertussis* and its properties for example as an adhesin, as well as a serum resistance factor might be easier to characterize.

It would also be of interest to investigate the mouse protection ability of purified recombinant BapC. This might have implications towards the use of BapC as an additional protective antigen in enhancing the protective efficacy of the new generation of acellular pertussis vaccines.

4.5 Role of BapC in susceptibility to antimicrobial peptides (AMPs)

It should be mentioned that lysis of Gram-negative bacteria by complement is due to the insertion of the C9 component of the membrane attack complex into the cytoplasmic membrane, which is analogous to the mechanism of killing by antimicrobial peptides in that lysis is also dependent on insertion of the peptide into the cytoplasmic membrane. Since BapC and BrkA proteins confer resistance levels to killing by complement, it was decided to examine and compare the susceptibility of their mutants to killing by antimicrobial peptides (AMPs).

In these experiments, the BP338 (BrkA⁻, BapC⁻) mutant was found to be more susceptible to killing by cecropin P1 than the single BrkA⁻ or BapC⁻ mutants ($P < 0.05$), but less susceptible than BP338 (Bvg⁻). The order of susceptibility to cecropin P1 in *B. pertussis* strains was approximately in the same order as serum sensitivity, suggesting a possible role for BrkA and BapC proteins in resistance to killing by some antimicrobial peptides and the possibility that the same mechanisms may be involved. Since BP338 (BrkA⁻, BapC⁻) was not as sensitive as the Bvg⁻ strain, this again suggests that *B. pertussis* factors other than BrkA and BapC can confer resistance to cecropin P1, just like resistance to complement. Modulated strains were not tested in these experiments, but testing these would help to support this hypothesis. Fernandez and Weiss (1996) found the same order of resistance to cecropin P1 with some of the same strains: BP338 > BP338 (BrkA⁻) > BP338 (Bvg⁻). They also reported that the sensitivity of the mutant strains to mastoparan was equal to that of

the *B. pertussis* wild-type strains, as found here. The susceptibility of BP338 (Bvg⁻) to the different antimicrobial peptides was consistent with the work of Fernandez and Weiss (1996), since it was markedly ($P<0.05$) less susceptible to protamine than other strains and had nearly equal susceptibility ($P>0.05$) to mastoparan compared to *B. pertussis* wild-type and BapC⁻ or BrkA⁻ derivative strains. In this study, the *B. pertussis* wild-type strains were more sensitive than their BrkA, BapC single or BrkA; BapC double mutant strains to killing by protamine. Overall, therefore, the evidence suggests that BapC, like BrkA, seems to confer resistance to at least one antimicrobial peptide, cecropin P1, but may possibly contribute to sensitivity to protamine.

AMPs are natural antibiotics that constitute a major part of the innate immunity of a wide range of organisms including humans. To date, more than 130 different AMPs have been characterised biochemically and have been shown to exhibit considerable variation both in amino acid sequence as well as in their secondary and tertiary structures. AMPs are small, most range from 14 to 40 amino acids in length, and polycationic amphipathic compounds (Reddy *et al.*, 2004). The most studied group of antimicrobial peptides includes linear, mostly helical, peptides (40 amino acids) that are toxic to bacteria only, such as cecropins isolated from the cecropia moth or magainins from the skin of frogs. Although linear peptides vary considerably in their chain length, hydrophobicity and distribution of charges, they share common motifs. Although the exact mechanism by which this family of antimicrobial peptides kills bacteria is not clearly understood, it has been shown that peptide-lipid interaction, rather than a receptor-mediated recognition process, plays a major role in their function. The essential role of AMPs is in the first line of defence against invading pathogens and their uncontrolled proliferation. It is believed that antimicrobial peptides, with very few exceptions, exert their activities to kill bacteria through interaction with the cytoplasmic membrane where they may cause leakage of cytoplasmic materials, and/or inhibiting membrane-associated functions like nutrient transport and energy transduction and/or cell lysis. It has been described that the membrane permeation by these

antimicrobial peptides occurs through one of the two general mechanisms: (A) transmembrane pore formation via a “barrel-stave” mechanism, or (B) membrane destruction/solubilisation via a “carpet-like” mechanism (Shai, 1999; Reddy *et al.*, 2004) such as in cecropin P1 (Yeaman and Nannette, 2003). Most of these peptides exhibit broad-spectrum antimicrobial activity in that they kill both Gram-negative and Gram-positive bacteria, and some are even effective at killing fungi or inactivating enveloped viruses (Reddy *et al.*, 2004).

There are some exceptions to this broad-spectrum activity: the animal-derived peptides such as magainin 2, are more effective at killing Gram-negative bacteria, whereas the insect-derived peptides such as mastoparan, tested against *B. pertussis* in the present study, are known to be more potent against Gram-positives. Although, there is agreement on the ability of these peptides to disrupt membranes, a specific target within the cytoplasmic membrane (or the cytoplasm) has not been demonstrated. However, in addition to the cytoplasmic membrane as the target of peptide activity, there has been reported that AMPs have a lethal effect solely through an interaction with the cell surface outside the cytoplasmic membrane, e.g., by disrupting the outer membrane of Gram-negative bacteria. Nevertheless, studies on the mode of action of magainin 2 and protamine have shown that disruption of the outer-membrane was not in itself sufficient to infer cell killing (Aspedon and Groisman, 1996). AMPs, with some exceptions carry a net positive charge although they may differ in their amino acid sequence and three-dimensional structure.

Although Gram-negative and Gram-positive bacteria differ in their cell envelope architecture, they are alike in that their cell surfaces usually carry a net negative charge. Thus, the initial bacterium-peptide interaction is an electrostatic attraction between the positively charged peptide and a negatively charged cell surface. The acidic groups (e.g., phosphates) residing in the teichoic acids associated with the cell wall confer this net negative charge in Gram-positive bacteria. In Gram-negative bacteria, it is the

lipopolysaccharide (LPS) in the outer membrane that bears the negative charge. It is expected that a resistance mechanism employed by bacteria is a reduction in the net negative charge of the cell surface. This reduction, which is not the only way to resist to the peptides, is influenced by the substitution of negatively-charged groups in the cell envelope with neutral or positively-charged moieties (Yeaman and Nannette, 2003). Stabilisation of the LPS in the outer-membrane bilayer so that there is less exposure to displacement by the peptide is another mechanism to be employed by the cell to reduce the uptake of AMPs. Mg^{+} serves to stabilise the LPS, and ultimately outer membrane integrity, by bridging adjacent LPS molecules through ionic association with phosphate residues on the lipid A core. Disruption of outer membrane integrity by AMPs is achieved through displacing Mg^{+} from the LPS and placing themselves into the outer-membrane bilayer so that it becomes permeable to the peptides or other compounds such as detergents.

Another type of intrinsic resistance to antimicrobial peptides can be achieved by peptide and potassium transporters, as described by the *sap* (sensitive to antimicrobial peptides) regulon in *Salmonella* spp. The Sap system is thought to mediate resistance to AMPs by transporting them into the cytoplasm (and away from their putative membrane targets) where they are either degraded by peptidases or can initiate a regulatory cascade resulting in the activation of the resistance determinants (Parra-Lopez *et al.*, 1993). BrkA and BapC are not like the Sap proteins in terms of sequence comparisons (Appendix AII.4 and AII.5) and so presumably do not operate in the same way.

BrkA and BapC, because of their predicted location on the surface of the bacterium and by the predicted negative charge (pI 5.89 for BrkA and pI 5.13 for BapC) of their processed forms, may be involved as barriers in the outer membrane to prevent puncture by the cationic cecropin P1 peptide. The resistance of the avirulent *bvg* mutant strain BP347 to killing by protamine compared to the susceptibility of the wild-type, *brkA*, *bapC*, or *brkA;bapC* mutants is interesting. The presence of a crystalline porin structure which is

unique to the avirulent form of *B. pertussis* (Kessel *et al.*, 1988), or the product(s) of a *bvg*-repressed gene might be possible explanations. In brief, *B. pertussis* strains appear to exhibit a broad range of susceptibility to different kinds of AMPs. For one of these peptides, cecropin P1, the wild-type *B. pertussis* strains were relatively more resistant to killing compared with the *brkA*, *bapC* or *brkA;bapC* mutants and *bvg* mutant strain, and this may reflect a possible role for BrkA and BapC in the pathogenesis of whooping cough via resistance to killing by some AMPs in the human respiratory tract. However, it would be of greater interest to examine those peptides such as tracheal or lung AMPs (the effector molecules of the innate respiratory immune systems, expressed constitutively in the human adult and newborn lungs and trachea, such as human beta-defensin, hBD-1) that are present in the human respiratory tract, which is the colonisation site by *B. pertussis*. However, commercial products were not available at the time of the experiments. A porcine beta-defensin 1 (pBD-1), which is the only well-studied beta-defensin with significant homology to human beta-defensins, was found to demonstrate resistance against infection of the upper respiratory tract with *B. pertussis* in older piglets. Furthermore, treatment with pBD-1 conferred complete protection of newborn piglets from intrapulmonary infection with *B. pertussis* (Elahi *et al.*, 2006). Another way of characterizing the role of BapC in resistance to AMPs, as well as its role in serum resistance, would be to study the recombinant form expressed in *E. coli* pET33b (BapC), away from other *B. pertussis* factors.

Although most studies of antimicrobial peptides have focused on their individual action on antimicrobial activity, it is apparent that AMPs function together with other components of the innate and adaptive immune systems. Recent data suggest that not only chemokines and other chemoattractants but also α -defensins, β -defensins display chemotactic activity for various cell types (Yang *et al.*, 2001). Neutrophil α -defensins are also chemotactic for immature dendritic cells, for resting CD4⁺ T cells, and for CD8⁺ T cells (Yang *et al.*, 2001). α -defensins also regulate processes such as complement activation, fibrinolysis,

mast cell degranulation and regulation of endogenous cortisol production (van Wetering *et al.*, 1999).

4.6 Effect of BapC protein on virulence of *B. pertussis* for mice

The development of pertussis infection is thought to begin with entry of *B. pertussis* into the respiratory tract of a susceptible host with the help of a variety of surface-exposed virulence-related proteins that allow its attachment to cilia in the respiratory mucosa. The colonisation of the ciliated epithelial cells in human respiratory tract is mediated by attachment proteins such as fimbriae, pertactin, tracheal colonisation factor, pertussis toxin and filamentous haemagglutinin. Evasion of host defence is a second requirement, which is presumed to be facilitated by adenylate cyclase toxin, pertussis toxin and serum resistance factor (BrkA) and finally local and systemic effects are mediated by tracheal cytotoxin and pertussis toxin, respectively. These *B. pertussis* virulence factors, except TCT, are coordinately regulated by the *bvg* locus, a two component regulatory system (Smith *et al.*, 2001).

Most of the autotransporter proteins in bacteria studied to date that have been characterized are associated with virulence (Lipski *et al.*, 2007). The potential role of BapC autotransporter protein in virulence of *B. pertussis* in a mouse model of infection was of interest since the BapC amino acid sequence revealed similarity to protein sequences of other, better-characterised, autotransporter proteins namely pertactin and BrkA. Moreover, a previous study (Bokhari, 2002) had suggested that a *bapC* mutant of the Taberman I strain of *B. pertussis* was less able to persist and colonise the trachea and the lungs of mice compared to its parent strain, which suggested that BapC, like pertactin and BrkA, might have a role in colonisation of the respiratory tract of the mouse by *B. pertussis*. A *B. pertussis* PRN mutant was cleared more quickly from the lungs of intranasally-infected

mice than its wild-type strain (Khelef *et al.*, 1994) and a *B. pertussis brkA* mutant was found to be 10-fold less virulent in colonising the respiratory tract (lungs) in intranasally challenged infant mice than the parent strain (Fernandez and Weiss, 1994).

In the present study, isogenic mutant and wild-type strains were compared in competition assays to quantify the virulence of individual strains. Preliminary tests with simple mixtures of two strains, wild-type and mutant, in a ratio of 1:1, and viable counting on plates showed no differences from the expected counts, which indicated no *in vitro* effect of one strain on the other.

When mice were infected intranasally with these mixtures, the *bapC* mutant of BP338 and the BP338 (BrkA⁻) strain were less able to persist in the lungs of mice compared to the parent strain, BP338, a nalidixic resistant-derivative of Tohama. Previously the same *B. pertussis brkA* mutant had been found to be 10-fold less virulent in colonising the respiratory tract (lungs) than the parent strain (Fernandez and Weiss, 1994). Thus, in the present study, the BrkA mutant of BP338 was less able to persist in the lungs of mice but not by as much as 10-fold. This difference from the work of Fernandez and Weiss (1994) may be a reflection of the different age of mice used for the infection and possibly mouse strain differences.

The data also showed that the BapC mutant of BP338 was less able to survive in the lungs compared to the wild-type and even compared to the BrkA mutant of BP338, suggesting that it was less virulent than either strain. The BrkA, BapC double mutant of BP338 showed a significantly lower ($P<0.05$) survival compared to the wild-type and both single mutants of BrkA or BapC, but it was not as poorly virulent as BP347 (Bvg⁻), which is what might be expected as the Bvg⁻ mutant is defective in many virulence-associated factors which would still be expressed in BapC and BrkA mutants. It is well-known that BP338 (Bvg⁻) has greatly reduced virulence in the mouse model compared to parent strain (Elder

and Harvill, 2004). It would seem clear, however, that both BapC and BrkA contribute to mouse virulence of *B. pertussis*.

The order of virulence determined by the competition assays was: BP338 (W/T) > BP338 (BrkA⁻) > BP338 (BapC⁻) > BP338 (BrkA⁻, BapC⁻) > BP347 (Bvg⁻), which is approximately the same as the order of resistance to serum killing; BP338 (W/T) > BP338 (BapC⁻) ≥ BP338 (BrkA⁻) > BP338 (BrkA⁻, BapC⁻) > BP347 (Bvg⁻) and to the antimicrobial peptide (cecropin P1); BP338 (W/T) = BP Taberman (W/T) > BP338 (BapC⁻) = BP338 (BrkA⁻) > BP338 (BrkA⁻, BapC⁻) > BP347 (Bvg⁻). Taken together, these findings suggest that BapC, like BrkA is an important virulence determinant of *B. pertussis* and also that the resistance to complement and antimicrobial peptides may have important roles in virulence. It is also possible that BapC may function as an adhesin, as indicated by the preliminary work of Bokhari (2002) but this needs further investigation.

However, these findings should be treated with caution since they may not reflect the natural behaviour of *B. pertussis* in man and also the initiation of infectious in mice requires relatively large challenge doses, which may alter the course of infection since smaller doses may colonize the respiratory tract of man in a different way.

4.7 Sequence of *bapC* gene in different *B. pertussis* strains and other *Bordetella* species

Recently, the gene encoding BapC protein was reported as a pseudogene by Sebaihia in 2006 with two frame shifts, one in the homopolymeric tract (HPT) of 13 cytosine (polyC) and another the in homopolymeric tract of 11 guanine residues at 5'-end of the α-domain of *bapC* of *B. pertussis* Tohama (locus_tag BP2738). A phase variable poly(C) was also reported in *B. parapertussis* 12822 and probably in *B. bronchiseptica* RB50, locus_tags BPP2591 and BB2033, respectively. *B. bronchiseptica* was predicted to express the full length BapC protein of 998 amino acids, whereas the products from *B. pertussis* Tohama

and *B. parapertussis* strains were predicted to be truncated (Henderson *et al.*, 2004). Previous work from this lab (Blackburn, 2000 and Bokhari, 2002) and this present study, however, suggested that BapC was produced by *B. pertussis* Taberman and the Glasgow University strain of Tohama. To address this apparent discrepancy, the 5'-end of the *bapC* gene was sequenced from chromosomal amplicons of a selection of *B. pertussis* strains and the nucleotide and deduced amino acid sequences were aligned. Sequencing was limited to the regions with the poly(C) and poly(G) tracts of *bapC* gene where variations had been observed in the *Bordetella* cluster (*B. pertussis*, *B. parapertussis* and *B. bronchiseptica*).

Analysis of the regions spanning the poly(C) and poly(G) tracts at the 5'-end of the *bapC* gene in *B. pertussis* strains revealed polymorphisms varying from 13-15 (C) and 9-12 (G) nucleotides. However no difference was found between the numbers of (C) nucleotides in BP Taberman (I), BP Tohama (Glasgow University strain), BP PICU, BP338 and BP338 (BrkA⁻) strains compared with the published sequence in the database without the frame shift (locus_tag BP2738). The 5'-region upstream to the homopolymeric (C) tract in BP PICU, BP Taberman (L1) and BP Tohama I strains exhibited 12-amino acid in-frame shift.

A slightly higher degree of variability was observed in the second sequence repeat (polyG) of the *bapC* gene in *B. pertussis* strains. Analysis of the poly(G) homopolymeric tract showed a polymorphism which varied from 9(G) in BP Tohama (Glasgow University strain) to 12(G) in BP338 strain. The multiple alignment of deduced amino acid sequences was interesting as this revealed an additional in-frame amino acid glycine in the chromosomal amplicons of *B. pertussis* strains apart from BP18323, a standard *B. pertussis* strain. An in-frame 11-amino acid shift downstream to the poly(G) region in BP PICU strain was observed, a probable consequence of two additional (G) nucleotides compared with the database.

Gogol *et al.* (2007) examined a collection of 90-geographically separate-isolates of *B. pertussis* for phase variation at HPTs including those in *bapC*, and found (G) alleles varying from G8 to G12 using colony PCR/LDR (polymerase chain reaction / ligase detection reaction), an ideal method for detecting rare genotypes within a mixed sample. In most of these cases, mixed allelic content was verified by sequencing of individual strains (Gogol *et al.*, 2007). The HPTs in *BapC* varied widely across the strain collection and allelic polymorphisms were detected even within a single round of culture (10^9 bacteria grown from a single colony). The predominant *bapC* HPT allele in 76/90 isolates was G11 but even some of these isolates contained a detectable fraction of both G11 and G12 alleles. Six strains carried primarily the G10 allele, two strains the G9 and four the G8 allele. Gogol *et al.* (2007) noted that the G11 allele, as in the Tohama genome sequence, causes a shift in the reading frame, predicted to result in a truncated *BapC* protein whereas a single base contraction would restore the reading frame. Similar allelic contractions and expansions can occur in the poly(G) tract of the *tcfA* gene in *B. pertussis* and in recombinant *E. coli* strains, leading to polymorphisms and phase variation (Gogol *et al.*, 2007; van Gent *et al.*, 2007).

In a study performed by Packard *et al.* (2004), polymorphism was found in five genes examined, *prnA*, *ptxA*, *ptxC*, *tcfA* and *fim2*, in a large number of *B. pertussis* isolates from the UK collected between 1920 and 2002. No polymorphism or sequence variation was found in the *brkA*, *bapC*, *cyaA*, *vag8* and *ompQ* genes; except a single nucleotide difference (a silent SNP) at position 2601 (2159 based on Henderson's nucleotide sequence of *bapC* gene) in *bapC* of all nine isolates. However, the five primer sets used for *bapC* only covered nucleotides 661-2990 (position 219-2548 based on Henderson's nucleotide sequence of *bapC* gene), starting downstream of the two potential phase variable homopolymeric C and G nucleotides tract.

A premature stop codon in *bapC* of *B. pertussis* Taberman (I) could be as a result of a frameshift in the homopolymeric (G) nucleotide tract upstream to this stop codon and consequently the disruption of the coupling of transcription and translation and eventually terminating the translation or could simply be result of a mismatch or error in PCR reaction. Expansion or contraction of the number of nucleotides in multiples of three, such as in *B. pertussis* BP 18323 (Table 3.4), could cause a size polymorphism of the protein or intriguingly change the function. AhpC protein of *E. coli* was described as having a simple reversible triplet expansion event in its coding sequence which altered function from a peroxiredoxin to a disulfide reductase in response to stress conditions (Ritz *et al.*, 2001).

It can therefore be concluded from the above observations that, although the entire *bapC* gene was not sequenced in these different *B. pertussis* strains, there are some minor nucleotide and amino acid changes in some strains but it is likely that all would be able to produce some form of BapC, unlike the *B. pertussis* Tohama genome strain. Thus, the data supported the findings by Blackburn (2000) and Bokhari (2002) that *B. pertussis* Tohama (Glasgow strain) and Taberman produce BapC protein.

Repetitive DNAs are common in eukaryotic cells, and are frequently located in extragenic regions. They are also becoming increasingly recognised in prokaryote genomes. Repetitive DNAs consist of simple homopolymeric tracts of a single nucleotide type [poly(A), poly(C), poly(G) or poly(T)] or a group of large or small numbers of several multimeric classes of repeats. These simple sequence repeats are involved in various mechanisms of gene regulation. Multimeric repeats are composed of identical units (homogeneous repeats) such as (PQP)₅ in pertactin, mixed units (heterogeneous repeats), or degenerate repeat sequence motifs. Monomeric sequences of variable length are tandem repeats, also called simple sequence repeats (SSRs). Their variability in repeat number is caused by slipped-strand mispairing, and is a mechanism for phase variation in e.g. fimbrial variation of *B. pertussis*. Mismatching of the neighbouring repeats in the tertiary

structure of DNA, results in insertion or deletion of nucleotides repeats during duplication mediated by DNA polymerase (Coenye and Vandamme, 2005). Those simple sequence repeats with longer monopolymeric nucleotides are more susceptible to slipped-strand mispairing and consequently show more mutability in their length than the shorter homopolymeric nucleotides. More presentation of A and T nucleotides in sequence repeats might possibly be explained by the fact that these nucleotides need less energy than C and G nucleotides for strand separation in slipped-strand mispairing (Coenye and Vandamme, 2005). SSR loci have high mutation rates, ranging from 10^{-3} to 10^{-6} per generation (Xu *et al.*, 2000). This high mutability is thought to play an important role in genome evolution via creating and maintaining genetic variation (Tautz *et al.*, 1986).

It has been shown that the number of mononucleotide repeats is correlated with genome size and GC content. Morgante *et al.* (2002) found that the overall SSR abundance was proportional to the genome size. In *B. bronchiseptica*, *B. parapertussis* and *B. pertussis* with 5.3, 4.7 and 4 million nucleotides, there are 7041, 6134 and 5684 mononucleotide repeats, respectively (Coenye and Vandamme, 2005). Intrinsic instability is one of the hypotheses for environmental selection to facilitate the process of evolutionary change by exploiting these “adjustable tuning knobs”. These repeats may reflect a strategy to cope with stresses (Rocha *et al.*, 2002) and may have enormous potential as major sources of genomic and phenotypic diversity (Rocha, 2003).

It is thought that the limitation of genetic diversity in many pathogens such as *Bordetella* species might be overcome by high frequency phase variation to adapt to the hostile and changing host environment. Reversible expansion or contraction of homopolymeric tracts (HPTs) are one of the most common mechanisms of phase variation (Gogol *et al.*, 2007) to evade the immune system.

Variation in numbers of sequence repeats is also one possible mechanism of regulation of gene expression via altering the conserved spaces between important domains such as -35 and -10 promoter regions and in the evolution of gene regulation (Huang *et al.*, 2003). It has been discovered that a stretch of ~15(C) nucleotides in the promoter region of the *fim* genes in *B. pertussis* is responsible for fimbrial phase transitions, by small insertions or deletions of 1-5 bp in this C-rich region (Willems *et al.*, 1990). This affects the distance between the putative -10 box and the transcriptional start site. By this means, *B. pertussis* strains can switch on and off the expression of the fimbrial types 1, 2 and 3 independently, so that strains can vary between serotypes 1; 1, 2; 1, 3; 1, 2, 3. However, the activity of these promoters and consequent gene expression may be more subtly regulated by HPT expansions and contractions rather than a threshold of bases between the activator binding region and the -10 box (Gogol *et al.*, 2007) as the HPT regions (such as C stretch regions) are hotspots for small insertions (duplications) or deletions due to transient misalignment during replication (Streisinger and Owen, 1985). Differences in DNA bending due to repeat sequences affecting DNA topography can also influence promoter strength and ultimately change gene expression (Perez-Martin *et al.*, 1994).

Another possible mechanism of regulation of gene expression by gaining or losing simple sequence repeats, a *recA* independent mechanism, is to change the integrity of ORFs (open reading frames) and result in phase variation via shifting in and out of frame (depending on the start of translation), which results in on-off switching of the gene product. This is more like the BapC situation, than fimbrial variation, since the Cs and Gs are not in the promoter region. The possibility of switching BapC on and off, according to the data of Gogol *et al.*, (2007), could be a means of antigenic variation in *B. pertussis* (and possibly other *Bordetellae*) in order to stay one step ahead of the host immune system (like fimbrial variation-alteration of adhesins). Both BrkA and BapC appear to confer serum resistance, and *B. pertussis* appears to switch BapC off and on very readily. No such mechanism has been reported for BrkA so even the BapC mutants will have some residual serum

resistance, as shown in the present study. However, it has been shown by real-time RT-PCR that the level of expression of the *brkA* gene and the relative amounts of *brkA* transcripts at different time points are strain dependent (Stefanelli *et al.*, 2006).

In future studies on BapC expression in different strains and species, in terms of observing frequent phase variation at the homopolymeric tract (HPT) in the coding region of the *bapC* gene, it would be advisable to check representative colonies of each strain or species, to determine whether BapC is expressed. Also, BapC expression could be quantified by real-time RT-PCR, then the sequence of the *bapC* gene in the individual clones analysed. Selection of phase variable poly(C) or (G) in *B. pertussis* strains could be evaluated by sequencing stored human isolates or studied *in vivo* by inoculating *B. pertussis* strains with variable poly(C) or poly(G) tracts in mouse and then direct sequencing of DNA from tracheal or lung samples taken 1-2 weeks after inoculation. The differences of these two repeated regions (C and G) could also be analyzed in *B. parapertussis* isolates of human and sheep origin and *B. bronchiseptica* isolates of animal and human origin, to examine any host specificity with respect to these *Bordetella* species.

Evidence for the presence of the *bapC* locus or homologous sequences was detected in the genome of other *Bordetella* species using DNA hybridisation with a *bapC* probe. It showed and confirmed the presence of *bapC* in *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* and this agrees with our knowledge of the genomes of these three species. However, no evidence of the *bapC* or any orthologous gene was found in the other *Bordetella* species examined, reflecting the known restricted genetic diversity between these three closely-related organisms (Parkhill *et al.*, 2003; Preston *et al.*, 2004; Sebahia *et al.*, 2006).

4.8 Comparative analysis of BapC with BrkA and pertactin

Bioinformatics is the combination of biology and information technology. Bioinformatics incorporates the development of databases to store and search data, and of statistical tools and algorithms to analyze and determine relationships between biological data. To represent nucleic acid or protein sequences, the conventional FASTA format is generally employed. Multiple sequence alignment can be performed to illustrate relationships between two or more sequences, and it can reveal many clues regarding protein structure and function.

The theory underlying a sequence similarity search is that similar sequences are likely to be homologous and therefore to have similar functions. However, any pair of DNA sequences may show some degree of similarity; sequence alignment is the first step in quantifying this in order to distinguish between chance similarity and real biological relationships and can show the differences between sequences as changes (mutations), insertions or deletions. Protein sequence alignment can reveal more distant evolutionary relationships than comparison of nucleic acid sequences (Eddy, 1998). Multiple alignment is more informative than pair-wise sequence alignment concerning evolutionary conservation since, when a residue is conserved in a family of sequences, it indicates that the residue might play a key structural or functional role (Eddy, 1998).

4.8.1 Protein comparison

The databases were searched for the best homologies or similarities with BapC using BLAST and FASTA (SwissProt). The best match for BapC was the *Bordetella* outer membrane protein pertactin which had also been shown for BrkA (Fernandez and Weiss, 1994). Although BrkA and BapC are 100 and 80 amino acids larger than pertactin, respectively, BrkA was found to be 29% identical and 40% similar to pertactin over a

common stretch of about 910 amino acids and BapC was 36.8% identical and 59.4% similar to pertactin over the same amino acid length, using Needle (global) pairwise alignment algorithms with Blosum62 matrix (<http://www.ebi.ac.uk/emboss/align/index.html?>). Moreover, BrkA and BapC exhibited 30.8% identity and 56.2% similarity in their whole amino acid sequences using the same tool and parameters.

BapC was previously predicted to be a protein of ~79.5 kDa according to the sequence data of Blackburn (1998) and Henderson (2000). Available *B. pertussis* genome sequence published in 2003, allowed prediction of signal sequence and the consequent actual BapC protein. The predicted molecular weight of BapC using ExPASy ProtParam tool (section 2.14) revealed a 104 kDa protein with an α -domain of 71 kDa after cleaving the signal sequence and C-terminus. However, the anti-BapC serum reacted with the whole cell fractions of *B. pertussis* strains at around ~90 kDa in immunoblotting experiments. This anomaly can be compared with the anomalous mobility of PRN on SDS-PAGE, whose processed passenger domain is actually 61 kDa, but runs at 69 kDa on SDS-PAGE. However, the anti-BapC serum also cross-reacted to some extent with the BrkA protein (~73 kDa) in the whole cell and outer membrane-enriched fractions of *B. pertussis*, which might be due to the existence of common epitopes.

A comparison of the noticeable features of BapC with BrkA and pertactin determined using CLUSTALW [<http://www.ebi.ac.uk/clustalw>] and MUSCLE (Multiple Sequence Comparison by Log-Expectation) [<http://www.ebi.ac.uk/muscle>] tools (Fig. 4.1). Jalview version 2+, a multiple alignment editor, was used to edit alignments and for the analysis of sub-families, prediction of functional sites and analysis of the conservation patterns.

Figure 4.1 Alignment of protein sequences of BrkA, BapC and pertactin

pertactin	-----MNMSLSRIVKAAP--LRRITTLAMALGALGAAPAAHADWNNQ-----	39
BapC	-----HNRKSNSESPAARYLRIGVVAVAGVAAGALPSSDVTQAAPAAAEVAKIEALS	54
BrkA	MYLDRFRQCPSSLQIPBSAWLHALAAALALAGMARLAFAAAQAPQPPVAGAPHAQDAGQ	60
	: * : : * : . * : * . . . : :	
pertactin	--SIVKTGERQHGIHIQ--GSD-----PGGVRTASGTTIKV	71
BapC	DADIYSDYEHEHGIVMTPDGKDDYISYSAESGRPKPPPLLSNFKPLGNDVVAERIRVEV	114
BrkA	EGEFDHRDNTLIAVFDDGVGINLDDDPDELGETAPPTLKDIHISVEHKNPMASKPAIGVRV	120
	. : : : * : . : *	
pertactin	SGRQAQGILLENPAAEQLFRNGSVTSSGQLSDDGIRFLGTVTVKAGKLVADHATLAN--	129
BapC	HGDETLGVYVDSEHSLTVRSDSTIDAYGKPPSVDSFDYAGAAVYAGTLNIENSTVHHNY	174
BrkA	SGAGRALTLAGSTIDATEGGIPAVVRRGGTLELDGVTVAGGEGMEPMTVSDAGSRLSVR-	179
	* . : : * . . * : . . : : :	
pertactin	VGDTWDDD-GIALYVAGEQAQASIADSTLQGAGG-VQIERGANVTVQR-----	175
BapC	AAQPFEDAVGVGVTSLGDKAILNVTDSEVSGARG-AVIGWGGEATFTD-----	221
BrkA	GGVLGGEAPGVGLVRAAQGGQASIIDATLQSILGPALIADGGSISVAGGSIDMDMGPGFP	239
	. : * : : . : . . : * : : . * . * * . . :	
pertactin	---SAIVDGGHLHIGALQSLQPEDLPPSRVVLRDNTNVTAVPASGAP-AAVSVLGASELTLD	231
BapC	---SVLRGSAGFLYAEMCDTCRDDDGTSPIRVQGGVVQGGMGANNVAVVATGSGKVAIE	278
BrkA	PPPPPLPGAPLAHPPLDRVAAVHAGQDGKVTLREVALRAHGPAATGVYAYMPGSEITLQ	299
	. : . . : . : : . : . : . : . : . : . : . : . : . : . : . : . :	
pertactin	GGHITGGR-----AAGVAAMQGAHVHLQRTATIRGDAAPAGGAVPGGAVP---GGAVP	282
BapC	NAELLGASGMYATFGAQVDMKGGRILAHNTNILGSQGYADGPYGGVVVTE--DGQVNLEG	336
BrkA	GGTVSVQGGDAGVVGAGLLDALPPGGTVRLDGTTVSTDGANTDAVLVRGDAARAEEVVN	359
	. . : . . : . . : . . : . : : . * . . : . . . :	
pertactin	FPGGGFGPVLDGWYGVVDSGSSVELAQSIIVEAPELGAAIRVGRGARVTVSGGSL	342
BapC	AKVSATGLGAAGLWLLGDKDTSPRASLRNTDVHGEVAAIALGFNGEANISGGSLSVEDGA	396
BrkA	TVLRTAKSLAAGVSAQHGRVTLRQTRIETAGAGAEISVLGFEPQSGSGPASVDMQGGG	419
	* : . : . : * . . . * : . :	
pertactin	VLETGG-----ARRFAPQAAPLS---ITLQAGAHQKALLYRVLPPEVKLT	387
BapC	VLTTLTTPDAVEYYYDYALSMEHLPADAPLTPVRVTLSDGARASGETLIAHGGLLPMTLRL	456
BrkA	ITTTGN-----RAAGIALTHGSARLEGVAVRAEGSGSSAAQLANGTLVVSAGSL	468
	: * : . . : : . . . : : . *	
pertactin	TGGADAQGDIVATELPSIP---GTSIG-----	411
BapC	SSGVDAARGDIVTLPPSAPP---DSAEQPDAAEPDAALEPDAAAQSDAKANARVMAQVD	512
BrkA	ASAQSGAISVTDTPCLKMPGALASSTVSVRLTDGATAQGGNGVFLQQHSTIPVAVALESG	528
	: : . * : :	

pertactin	-----PLDVALASQARWGTGATRAVDLSLSIDN-ATWVMTDNSNVGALRL	453
BapC	GGEPVAVPIPAESHDPADIDVFIDSGAQWRGMTKTVNALRIED-GTWTVTGSSTVNSLHL	571
BrkA	ALARGDIVADGNKPLDAGISLSVASGAAWHGATQVLQSATLGKGGTWVNVNADSRVQDMSM	588
	:.: : * * * * *: : : : : . * . * . * * : :	
pertactin	ASDGSVDFQQPAEA-GRFKVLTVNLAGSGLFRMNVFADLGLSDKLIVMQDASGQHRLWV	512
BapC	QA-GKVAYATPAESDGEFKHLRVKTLSGSGLFEMNASADLSDGDLVVVSDEASGQHKVLV	630
BrkA	RG-GRVEFQAPAPE-ASYKTTLTQTLDGNGVFLNTNVAAGQNDQLRVTGRADGQHRVLV	646
	. * * : * * . : * * : : * * * . * : : . * * * * * . * * * : : *	
pertactin	RNSGSEPAS-ANTLLLVTPLGSAATFTLANKDQKVDIGTYRYRLAANG-NGQWSLVGAK	570
BapC	RGAGTEPTG-VESLTLVELPEGSQTKFTLANRGGVVDAGAFRYRLTPD--NGWGLERT-	686
BrkA	RNAGGEADSRGARLGLVHTQGGNATFRLANVGKAVDLGTWRYSLAEDPKTHVWSLQKAG	706
	* : * * . * * * . : * * * * . * * * : * * : : . * * :	
pertactin	APPAPKPAPQPGPPPPPPPPPEAPAPQPPAGRELSAAANAAVNTGGVGLASTLWYAES	630
BapC	-----SQLSAVANAAALNTGGVGAASSIWYAE	713
BrkA	-----QALSGAANAAVNAADL---SSIALAES	730
	* * . * * * . * : : : * : : * *	
pertactin	NALSKRLGELRLNPDAGGAWGRGFAQQQLDNRAGRRFDQKVAGFELGADHAVAVAGGRW	690
BapC	NALSKRLGELRLDPGAGGFWRGTFQAQQQLDNKAGRRFDQKVYGFELGADHAIAGQQGRW	773
BrkA	NALDKRLGELRLRADAGGPWARTFSERQQISNRHARAYDQTVSGLEIGLDRGWSASGGRW	790
	* * . * * * * * . * * * * * : : * * : * * * * * : * * : * * * * * : * * *	
pertactin	HLGGLAGYT RGD RGFTGDGGGHTDSVHVGGYATYIADSGFYLDATLRASRLENDFKVAGS	750
BapC	HVGGLLGYT RARRSFIDDGAGHTDSAHIGAYAAVADNGFYFDSTLRASRFENDFTVTAT	833
BrkA	YAGGLLGITYADRTYPDGGGKVKGLHVGGYAAVVDGGYYLDTVLRLGRYDQQYNIAGT	850
	: * * * * * . * : * * * : . . * : * * * : . * * * : * * * : * * * : * * *	
pertactin	DGYAVKGKYRTHGVGASLEAGRRFTHADGWFLFPQAE LAVFRAGGGAYRAANGLRVRDEG	810
BapC	DAVSVRGKYRANGVGATLEAGKRFTLHDGWFEVFPQSEVSLFHASGGTYRAANNLSVKDEG	893
BrkA	DGGRVTADYRTSGAAWSLEGGRRFELPNDWFAEPQAEVMLWRTSGKRYRASNGLRVKVDA	910
	* . * . * * : * . : * * * * : . * * * * : : : . * * * * * * : : .	
pertactin	GSSVLGRLGLEVGKRIELAGGRQVPYIKASVLQEFDGAGTVHTNGIAHRTELRGTRAEL	870
BapC	GTSAVLRLGLAAGRRIDLKDRVIQPYATLSWLQEFKGVTTVRTNGYGLRTDLSSGGAEL	953
BrkA	NTATLGRLGLRFGRRIALAGGNIVQPYARLGWTQEFKSTGDVRTNGIGHAGAGRHGRVEL	970
	. : : : * * * * * : * * * . . : * * . * * . . * : * * . * * *	
pertactin	GLGMAAALGRGHSLYASYEYKGPKLAMPWT FHAGYRYSW	910
BapC	ALGLAAALGRGHQLYTSYAYAKGNKLTLPWT FHLGYRYTW	993
BrkA	GAGVDAALGKGHNLYASYEYAAGDRINIPWS FHAGYRYSF	1010
	. * : * * * : * * * * * : * : : * * * * * * * :	

Signal sequence (peptide): Pertactin: 34 aa (grey), BrkA: 42 aa (Oliver *et al.*, 2003) (blue), BapC: 38 aa (this study, green) {45 aa predicted by Henderson *et al.*, (2004)}.

RGD motifs (green): Pertactin: 2, BrkA: 2, BapC: 1

SGXG motif (grey): Pertactin: 0, BrkA: 2, BapC: 1

Glu⁶⁰¹-Ala⁶⁹² (Blue-bold type): A conserved region within the *Bordetella pertussis* autotransporter BrkA, necessary for folding of its passenger domain (Oliver *et al.*, 2003).

Val⁴⁷²-Leu⁵⁶⁶ in pertactin: probably promotes folding of the passenger domain and is associated with protective immunity in a mouse aerosol model (Charles *et al.*, 1991).

Processing site: Asn⁷³¹-Ala⁷³² in BrkA (yellow), Asn⁶³¹-Ala⁶³² in pertactin (yellow), Asn⁷¹⁴-Ala⁷¹⁵ in BapC (with signal sequence) (blue).

Arg²⁶⁰-Gly²⁹⁴ in pertactin: RGD motif is in the T1 loop of pertactin

GGXXP₅ : a heterogenic amino acid repeats in pertactin (region 1)

Consensus symbols:

An alignment displays by default the following symbols denoting the degree of conservation observed in each column:

"*" means that the residues or nucleotides in that column are identical in all sequences in the alignment.

":" means that conserved substitutions have been observed.

"." means that semi-conserved substitutions are observed.

The most striking identities were found in the C-terminal ends (β -domain) of the three proteins. The BapC and BrkA proteins are 63% similar, BapC and pertactin are 79% similar, and BrkA and pertactin are 54.5% similar over their last 300 amino acids, as determined by BLASTp analysis (data not shown). They share a C-terminal outer membrane localisation motif (FHA/LGYRYS/TW/F), which consists preferentially of amino acid residues with hydrophobic side chains such as phenylalanine or tryptophan in the last nine C-terminal residues. The overall similarity of these β -domains is perhaps not surprising given that the function of these moieties is to form a β -barrel. Generally, the C-terminus domains of autotransporters are composed of 250-300 amino acid residues, which all show some homology but vary in their sequences (Henderson *et al.*, 2004). The amino acid sequence similarity of the passenger domains (α -domains) of BapC/BrkA and BapC/pertactin were 36.4% and 40.3%, respectively (data not shown).

With regard to the signal sequence in these proteins, recent analysis of BrkA using the SignalP v.2.0 hidden Markov model method, and recent experiments, have shown a processing cleavage site between residues Ala⁴² and Gln⁴³ (Oliver *et al.*, 2003). Henderson *et al.* (2004) have reported a 34 amino acid signal sequence for pertactin and a predicted 45 amino acid sequence for BapC. However, in this study, analysis of BapC in *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* using SignalP v.3.0 (released 2004) revealed a signal peptide with a maximum cleavage site probability between amino acid residues Ala³⁸ and Gln³⁹ (Figs. 3.49, 3.50, 3.51).

The transport mechanism of the passenger domain of the autotransporters across the outer membrane remains undefined, although four models of translocation such as hairpin, threading, multimeric and Omp85 (YaeT) have been proposed (see section 1.6.5). Kostakioti and Stathopoulos (2006) revealed that a strictly conserved 14-amino-acid motif (EVNNLNKRMGDLRD) within the predicted α -helical linker region, upstream of the β -domain of SPATEs (serine protease subfamily of autotransporters) is important for

passenger secretion across the outer membrane and confers polarity on the helical structure. Mutation in this region showed a severe effect on the secretion process and seven of these amino acid residues are essential.

The proteolytic processing site at amino acid 715 of BapC is located at the α -helical region of the N-terminus of the β -domain and sequence analysis of this region showed homologous or conserved sequences (EGNALSKRLGELRL) with the 14-amino-acid residues of the serine protease subfamily of autotransporters. It has been shown that this motif was also conserved with slight variations in other autotransporters such as *B. pertussis* BrkA and pertactin (Kostakioti and Stathopoulos, 2006). Sequential deletions or site directed mutagenesis of this region in BapC or other autotransporters could uncover the potential effect of this α -helical region in the efficiency of translocation.

It has been shown that a conserved region within the C-terminus of the BrkA passenger domain, upstream of the predicted β -domain, from glutamic acid (E) at position 601 to alanine (A) at position 692 (Fig. 4.1, blue bold-type sequences) is necessary for correct passenger folding (Oliver *et al.*, 2003) and functions as an intramolecular chaperone. The observation that this domain is conserved among other autotransporter proteins in Gram-negative species such as *E. coli*, *Shigella* or *Serratia*, which possess diverse functions, suggests a possible general role for this region. It was noted that residues corresponding to the conserved region of BrkA (amino acids 606 to 702) in pertactin (amino acids 472 to 566) were also located at the base of a β -helical structure (Oliver *et al.*, 2003a).

To identify regions of BapC homologous with the conserved domain in BrkA that might indicate a conserved function, a comparative analysis of this junction region in BrkA with BapC and pertactin using BLASTp (release 2.2.16) revealed 46% identity and 58% similarity between BrkA and pertactin; and 39% identity and 52% similarity between BrkA and BapC. Residues Phe⁵⁸⁸-Thr⁶⁷⁴ of the BapC passenger, which correspond to residues

Tyr⁶⁰⁴-Ala⁶⁹² of BrkA, are predicted (by Proteus Structure Prediction Server (release 2.0), a secondary structure prediction tool) to be accommodated at the base of a β -strand structure.

BapC, BrkA and pertactin proteins contain one, two and two arg-gly-asp (RGD) motifs respectively (Fig. 4.1) that may promote binding to integrins and are important in adhesion to mammalian cells (Wells *et al.*, 2007). However the spacing of the RGDs is not conserved between these proteins and, interestingly, the second RGD motif of pertactin appears downstream of the processing site in the β -domain, quite far away from the first one, and is not a part of the mature protein. The first RGD motif of pertactin is located in the T1 loop (Arg²⁶⁰-Gly²⁹⁴) (Fig. 4.1), which is one of the three turns separating the three parallel β -strands of the β -helix of pertactin. This T1 loop contains proline-rich repeats, GGXXP₅, named “region 1”, and is polymorphic in clinical isolates and vaccine strains (Mooi *et al.*, 1998) and also associated with protection in an animal model (King *et al.*, 2001). The RGD motif in BapC is located halfway between the predicted signal sequence and processing site, similar to the position of the second RGD in BrkA, (positions 418 and 490 in BapC and BrkA, respectively).

In pertactin, there are two PRRs (proline rich regions) that are widely present in proteins with binding activity (Emsley *et al.*, 1996). These protruding loop tandem repeat motifs interrupt the helical structure of the pertactin and provide a functionally important binding site. (GGXXP)₅ (starting at position 266, Fig. 4.1) is one of the PRR that appears directly downstream of the first RGD motif that probably mediates interaction with epithelial cells, and the other is the (PQP)₅ motif in the C-terminus of pertactin (starting at position 579, Fig. 4.1), which contains the immunodominant region of the protein and is a major immunoprotective epitope (Emsley *et al.*, 1996). Unlike pertactin, BrkA and BapC do not possess the (GGXXP)₅ and (PQP)₅ tandem repeats. However, BrkA and BapC do have two and one potential glycosaminoglycan attachment sites (SGXG), respectively, which have been suggested may be involved in the mechanism for serum resistance in *B. pertussis*

possibly by inhibition of C9 polymerization (Fernandez and Weiss, 1994). This motif is absent in pertactin.

These findings of conserved motifs within the α -helical region of autotransporters may provide a new tool(s) for the prevention and treatment of infectious disease as they could represent ideal antigens for vaccine development. Moreover, the critical requirement of certain residues within the 14-amino-acid passenger secretion motif of autotransporters makes them a potential target for novel antimicrobial peptides, as they could interfere with interactions necessary for the export or biogenesis of the secreted autotransporter virulence factors (Kostakioti and Stathopoulos, 2006). It has been demonstrated that the autotransporter proteins such as BrkA can be employed as surface display systems in vaccine strains for intranasal administration. Heterologous peptide (neutralizing peptide SP70 from enterovirus 71) was fused within the passenger domain of BrkA and expressed in the highly-attenuated vaccine strain BPZE1, and had potential ability to deliver heterologous vaccine antigens through the nasal route (Ho *et al.*, 2007).

4.8.2 Gene comparison

Putative BvgA binding sites have been found in a region between *brkA* and *brkB* along with a single copy of the sequence TTTCCT upstream of the *brkA* and two copies upstream of *brkB* (Fernandez and Weiss, 1994). A database search of the upstream flanking region of *bapC* using the *B. pertussis* Tohamal genome sequence (BLAST search at http://www.Sanger.ac.uk/projects/B_pertussis) indicated two potential BvgA binding sites (TTTCATA and TTTCGTA) upstream of the *bapC* gene at positions -230 bp and -144 bp, respectively (data not shown) from the start of the predicted signal peptide (contig BX640419) between a probable ammonium transporter gene (*amtB*, locus_tag BP2737) and the *bapC* gene. These two potential BvgA binding sites upstream of the predicted translational site of *bapC* are also similar to the characteristic heptameric sequence TTTC(C/T)TA identified by Kinnear *et al.* (1999) upstream of *prnA*. These sequences,

required for regulating the transcription of these genes through interaction with the RNA polymerase, are also located upstream of the Bvg-regulated *ptx*, *cya*, *fha* and *bvg* genes of *B. pertussis*.

In order to identify the true *bvg*-dependent transcriptional start site of *bapC* or to characterize the *cis*-regulatory sequence upstream of and in the *bapC* promoter, a series of plasmid-borne *bapC-lac* transcriptional fusions should be introduced into *B. pertussis* BP338, a derivative of Tohama I, by conjugation to localize the region that determines the *bvg*-dependent transcriptional activity from the *bapC* promoter. Such investigations have been done for the pertactin gene (Kinnear *et al.*, 1999). For further characterization of sequences necessary for *bapC* activation, the upstream region with *lac* fusions and with sequence alterations could be inserted into the chromosome of this strain. It will then be possible to determine the *bapC* promoter activity using β -galactosidase assays on the altered constructs. DNase I protection analysis could also demonstrate the BvgA binding site upstream of the putative *bapC* transcription site.

No obvious -10 and -35 promoter sequences or Shine-Dalgarno sequences were detected either in *brkA* (Fernandez and Weiss, 1994) or in the *bapC* gene. Although a transcriptional start site was identified by primer extension analysis (Graeff-Wohlleben *et al.*, 1995) in the *prnA* gene for pertactin, no σ^{70} consensus promoter was found upstream of this site. However, later genetic and biochemical analyses performed by Kinnear *et al.* (1999) showed the pertactin gene transcription start site as a cytosine residue located 125 bp upstream from the previously reported start site.

The promoter region of *bapC* could also be analysed to determine which class of *vag* it belongs to (early or late *vag*) by determining at what stage it starts to be transcribed compared with the other *vag* genes. This could be performed by the RT-PCR method similar to that described earlier by preparing RNA samples at intervals after switching the

culture from Bvg⁻ to Bvg⁺ phase. Alternatively, RNA preparations could be separated by electrophoresis in a formaldehyde agarose gel followed by transfer of the RNA to a nylon membrane and detecting the relevant RNA by hybridisation with a labelled probe. It could also be determined via fusion of the promoter region of *bapC* to the *lac* reporter function (or GFP or Lux reporters) to allow the time of promoter-activation to be determined.

A search for similarities to the *E. coli* known promoter sequences revealed either no or only weak homologies. Another attempt to search in the 5'-upstream of *bapC* gene using the Neural Network Promoter Prediction (NNPP) tool (version 2.2) (1999) (http://www.fruitfly.org/seq_tools/promoter.html) demonstrated two potential promoters upstream of the putative signal peptide with a more than 0.90 cutoff score. One of the predicted promoters overlaps with the first potential start codon (0.98 cutoff score) of the signal sequence and the other is located 100 bp upstream of the signal sequence start codon (0.90 cutoff score) just a few nucleotides downstream of the second potential Bvg binding site. Nevertheless, primer extension analysis could identify the transcriptional start site of the *bapC* gene. These two promoters could be consistent with the previous study performed by Bokhari (2002) which revealed the existence of another smaller transcript by RT-PCR, in addition to one of the expected size, in Taberman I (wild-type) that could have been the product of another promoter in regulating the production of the smaller transcript.

It should be taken into account that apart from the specific transcription factors, global mechanisms which change DNA topology or transcript stability via environmental stimuli such as different growth conditions can affect expression of virulence factors in *B. pertussis* (Graeff-Wohlleben *et al.*, 1995). It would therefore be of interest to investigate whether additional parameters are involved in the expression of BapC, in response to differences in the DNA topology such as supercoiling, which may be influenced by changing the *in vivo* conditions and provide an additional or complementary mechanism for regulation, as shown for the effect of supercoiling on expression of pertussis toxin

(Scarlato et al., 1993) or, in the case of the pertactin gene, in response to a DNA gyrase inhibitor where the effect was different compared to that on other *bvg*-regulated genes (Graeff-Wohlleben et al., 1995).

A *rho*-independent terminator sequence downstream of the translational stop codon of the *bapC* gene was found. This terminator adopts a hairpin or loop-shape secondary structure, consisting of a GC-rich stem-loop region followed by a run of U residues, which is considered responsible for RNA polymerase terminating mRNA synthesis. The downstream U-rich region destabilizes the RNA-DNA hybrid when RNA polymerase pauses at the terminator hairpin. A search upstream of the stop codon for a *rut* site (an acronym for *rho* utilization), a sequence within the transcript upstream of the site of termination for binding of the *rho* factor for *rho*-dependent termination which should be rich in C and poor in G that accounts for about half of *E. coli* terminators, showed only 32% C nucleotide and 26% G nucleotide in this region, that could not function as a site for the binding of *rho* factor.

In summary, the protein and gene comparative analysis between BapC, BrkA and pertactin clearly revealed the homology in their β -domains, as would be expected for autotransporter proteins but no clear homology in α -domains (passenger) to suggest common functions, despite BrkA and BapC apparently having a role in serum resistance and all three proteins possibly being involved in adhesion.

4.9 Modelling/prediction of BapC structure

Homology or comparative modelling is a robust technique for predicting or generating detailed three-dimensional structures of proteins based on the coordinates of known homologues found in the Protein Data Bank (PDB). In homology modelling, the quality of the model strongly depends on the degree of similarity between the query sequence and the

matching database sequence, with proteins sharing the highest degree of similarity being modelled best.

The Proteus structure prediction server 2.0, PROTEUS2 (<http://wks16338.biology.ualberta.ca/proteus2/index.jsp>), a web server designed to support comprehensive protein structure prediction and structure-based annotation, found a file (1DABA) (i.e. P.69/pertactin) homologous to BapC with an overall confidence value of 78.3%. The predicted helix content, beta sheet content and coil content were 4% (43 residues), 53% (527 residues) and 43% (423 residues), respectively (Fig. 4.2). The 3D structure of the PDB homolog (1DABA) (Fig. 4.4) related to BapC protein with E-value of 8.0 matches to residues 112-694 and can be seen with Webmol viewer in Java programme. Secondary structure prediction using PROTEUS2 also suggested a translocator domain in the C-terminus of BapC with 14 transmembrane anti-parallel β -strands and a transmembrane α -helix and forming a β -barrel with a central hydrophilic channel like other autotransporter proteins.

SWISS-MODEL (version 3.5), an automated comparative modelling server of three-dimensional (3D) protein structures, was used for the tertiary structure prediction of BapC via the web page (<http://swissmodel.expasy.org>) or directly as a link from SWISS-PROT entries on the ExpASy server. SWISS-PROT is primary database of confirmed protein sequences with annotations relating to structure, function and protein family assignment.

Structural data are maintained as text files using the PDB format devised by the Protein Data Bank and contain atomic co-ordinates together with annotation, comments and experimental details derived from X-ray crystallography and NMR studies (available at the following URL: <http://www.pdb.org/>).

Figure 4.2 Prediction of the secondary structure of BapC by Proteus tool

Summary:

- Sequence Name: **BapC protein**
- Number of residues read in: **993**
- Number of useable PDB homologs found: 1
 - [1DABA](#), e-value = 8.0E-45 THE STRUCTURE OF BORDETELLA PERTUSSIS VIRULENCE FACTOR P.69
- Overall confidence value: **78.3%**
- Predicted % Helix content: **4 % (43 residues)**
- Predicted % Beta sheet content: **53 % (527 residues)**
- Predicted % Coil content: **43 % (423 residues)**

Graphical Alignment of PDB Homologs:

QUERY
1DABA 112 594

Legend:

H = Helix

E = Beta Strand

C = Coil

Line 1 = sequence (single letter IUPAC code, 60 characters per line)

Line 2 = secondary structure (H, E or C)

Line 3 = confidence score (0-9, 0 = low, 9 = high)

A '*' character above the overall prediction indicates the homolog's structure was used at this residue.

Predicted Secondary Structure:

```

1                               60
MNDRKSNMSPAARYLRFQVAVAGVAAGALPSSDVDAQAAPAAAEVAKIEALSDADIYS
CCCCCCCCCCCCHHHHHHHCEEEEECCCCCCCCCCCCCCCCCCCCHHHHHHHHHECCCCCCCC
98778777764678874489987554456777788777765547888753367777777

61                               ***** 120
DYEHEHGIVMTPDGKDDYISYRSAESGRPKPPPPLSNFKPLGNDVVAERIRVEVHGDETL
CCCCCEEEEECCCCCEEEEECCCCCCCCCCCCCCCCCCCCCEEEEECEEEEECEEE
77887657886788875778866777666777766767777677876767899878877

121 ***** 180
GVYVDSEHRSLTVRDSTIDAYGKPPSVDSPDYYGAAAVYAGTLNIENSTVHHNYAAQPFE
EEEECCCCEEEECEEEEECCCCCCCCCCCCCEEEEEEEEEEEEECCCCCCCCCCCCC
9999688768998777588767766677776578998899889878865678777777

181 ** ***** 240
DAVGVGVTSLGDKAILNVTSEVSGARGAVIGWGGEATFTDSVLRGSAFGLYAEMCDTCR
CCCCEEEEEECCCCEEEEEEEECCCCCEEECCCCCEEEEEEEEECCCCCEEECCCC
777768898588779998777898888989977877788899998589678898667777

241 ***** 300

```

DDDGTSPSIRVQGGVVQGGMGANNVAVVATGSGKVAIENAELLGASGMYATFGAQVDMKG
 CCCCEEEEEECCCCEEEECCCCCCCCEEEECCCCEEEEECCCCCEEEEECCCC
 777758999976866885788887789997785789857578778788888898887688

301 ***** ***** ***** ***** 360
 GRILAHNTNIGSQGYADGPYGGVVVTEGQVNLEGAKVSATGLGAAGLWLLGDKDTSR
 CEEEECCCCCCCCCEEEEECCCCCEEEEECCCCCCCCCCCCCCCCCEEEEECCCC
 58997676565677565678888887788679977777757778878888768856556

361 ***** ***** 420
 ASLRNTDVHGEVAAIALGFNGEANISGGSLSVEDGAVLTTLTPDAVEYYDYALSMEHLP
 EEEEECCCCCCCCCEEEEECCCCCEEEEECCCCCEEEEECCCCCEEEEECCCC
 88999858888688999858757888999998887899767775656545676566567

421 ***** ***** 480
 ADAPLTPVRVTLSDGARASGETLIAHGGLPMTLRLSSGVDA **RGD** IVTLPPSAPPDSAEQ
 CCCCCEEEEEEEEECCCCCEEEEEEEEECCCCCEEEEECCCCCEEEEECCCCCCCC
 899888578988999899888889997788778888889888775789966788775676

481 ***** ***** 540
 PDAEPEPDALPDAQAQSDAKANARVMAQVDGGEPVAVPIPAPSHPDAPIDVFIDSGAQ
 CCCCCCCCCCEEEEECCCCCEEEEECCCCCEEEEECCCCCCCCCCCCCEEEEECCCC
 677766546656775777677667878867888788888877776777788898887889

541 ***** ***** 600
 WRGMTKTVNALRIEDGTWTVTGSSTVNSLHLQAGKVAYATPAESDGEFKHLRVKTL **SGSG**
 EEEEECCCCCEEEEEEEEECCCCCEEEEEEEEECCCCCCCCCEEEEEEEEECCCC
 9998889878998888899888987899887887799888889989899999999999

601 ***** ***** 660
 LFEMNASADLSDGDLVVSDEASGQHKVLVRGAGTEPTGVESLTLVELPEGSQTKFTLAN
 EEEEECCCCCEEEEEEEEECCCCCEEEEEEEEECCCCCEEEEECCCCCEEEEECCCC
 999999988898889999999999999999999888778778889998888999999

661 ***** ***** 720
 RGGVVDAGAFRYRLTPDNGVWGLERTSQLSAVANAALNTGGVGAASSIWYAEGN **ALSKRI**
 CCCCEEECCCCCEEEEECCCCCEEEEECCCCCCCCCCCCHHHHHHHHHHHHHHHHHHHH
 999899899999988888998756777766645788999999999999999998887

721 ***** ***** 780
GELRLDPGAGGFWRGRTFAQKQQLDNKAGRRFDQKVYGFELGADHAIAGQQGRWHVGGLLG
 HHCCCCCCCCCEEEEECCCCCEEEEECCCCCEEEEEEEEEEEEEEEEECCCCCEEEEE
 64578887766899987676565677666555557888999999987998578999999

781 ***** ***** 840
 YTRARRSFIDDGAGHTDSAIGAYAAAYVADNGFYFDSTLRASRFENDFTVTATDAVSVRG
 EEEEEEEEECCCCCEEEEEEEEEEEEEEEEECCCCCEEEEEEEEEEEEEEEEECCCC
 8766677567757776677777667655875676565666777777778766777677

841 ***** ***** 900
 KYRANGVGATLEAGKRFTLHDGWFVEPQSEVSLFHASGGTYRAANNLSVKDEGGTSAVLR
 CCCCCEEEEEEEEECCCCCEEEEEEEEEEEEECCCCCEEECCCCCEEEEECCCC
 666656899988777766787578765778899998577657567668997788678887

901 ***** ***** 960
 LGLAAGRRIIDLKDRVIQPYATLSWLQEFKGVTTVRTNGYGLRTDLSGGRAELALGLAAA
 EEEEEEEEECCCCCEEEEEEEEEEEEECCCCCEEECCCCCEEEEEEEEEEEEE
 7665555677478768888888999977885788576689986888589998999999

961 ***** ***** 993
 LGRGHQLYTSYEYAKGNKLTLPWT **FHLGYRYTW**
 ECCCCEEEEEEEECCCCCCCCCEEEEEEEEECCCC
 689779999988866777777667889999977

Figure 4.3 Model of the tertiary structure of the passenger domain of the BapC protein obtained using the SWISS-MODEL programme

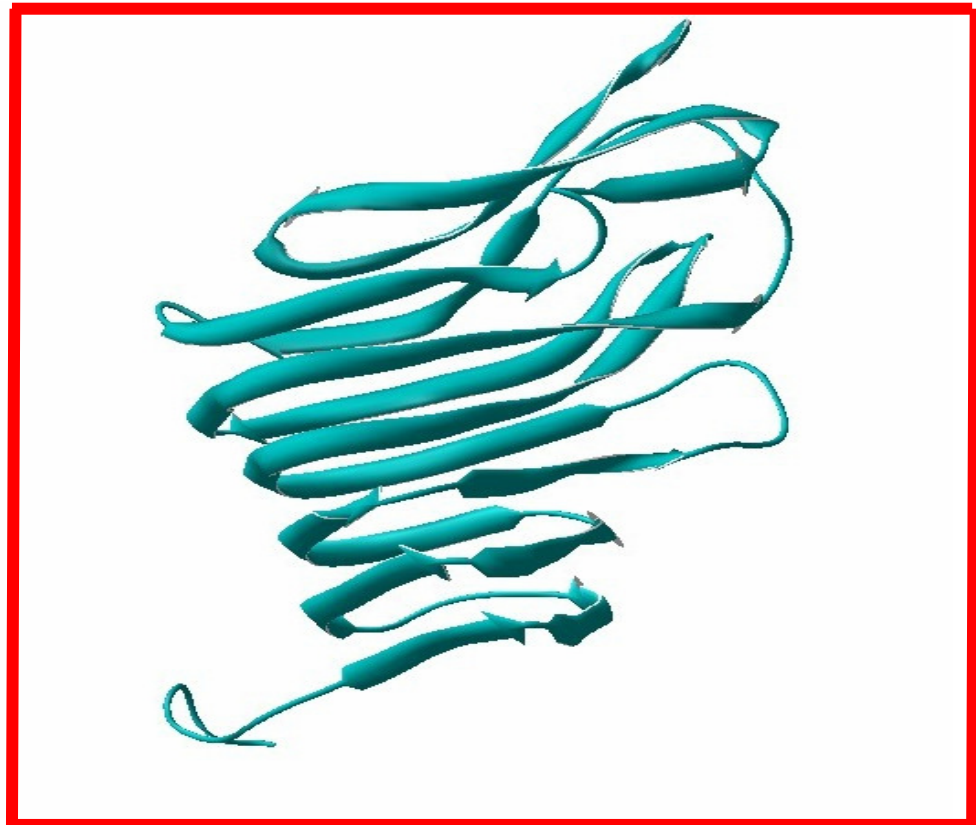
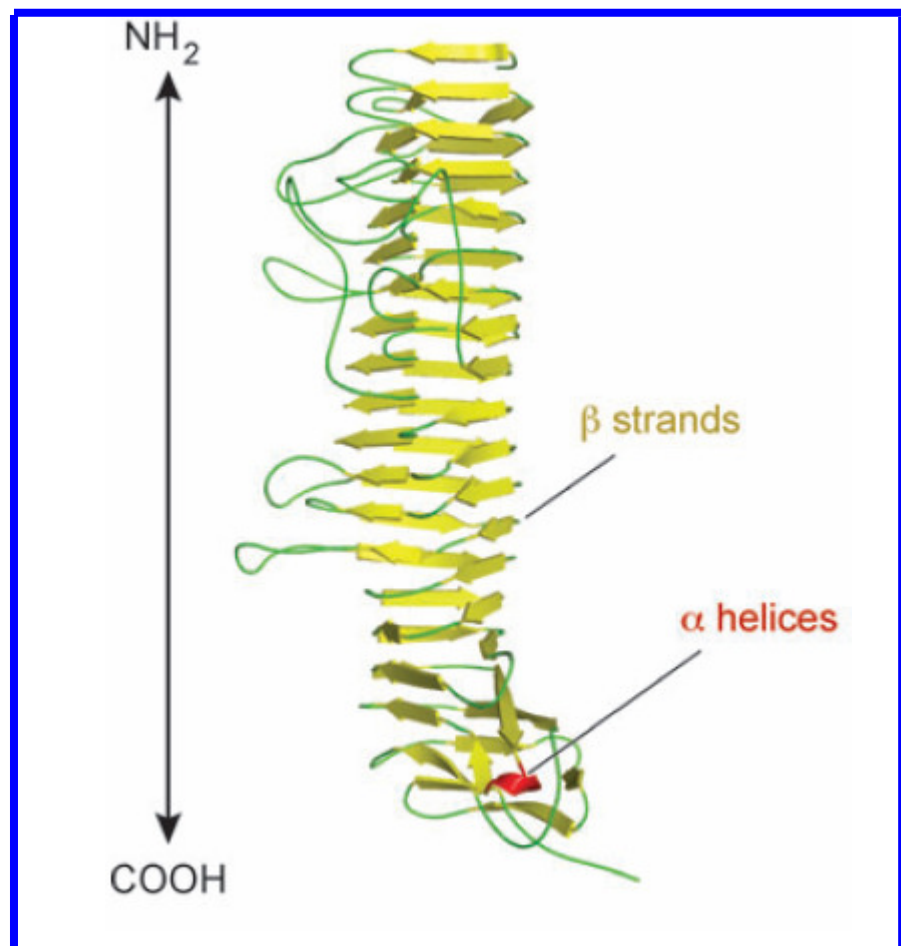


Figure 4.4 Tertiary structure of pertactin (Dautin and Bernstein, 2007)



The process for proteins of known sequence but unknown structure such as BapC, is first to retrieve homologous proteins via the ExPASy tool, (the most critical step in homology modelling) onto which one can subsequently align the sequence to build a preliminary three-dimensional model, and then the SWISS-MODEL server can build the final model based on the homology model. The pertactin PDB file based on the tertiary structure of PRN (Emsley *et al.*, 1996) was automatically employed as a template for BapC modelling and alignment done with more than 30% homology during the model building process. The deduced automated homology modelling of a tertiary structure of BapC passenger domain can be seen in Fig. 4.3. The tertiary structure of pertactin is seen in Fig. 4.4.

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6.6. Appendices

6.1 Appendix I

AI.1 Media composition

AI.1.1 Luria Bertani broth (LB)

1 litre

Tryptone (Duchefa Biochemie)	10 g
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Yeast extract (Duchefa Biochemie)	5 g
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Sodium chloride	10 g
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Add 1.2% microagar (Duchefa Biochemie) for making solid media and then autoclave at 15 p.s.i. for 15 min.

AI.1.2 Bordet Gengou agar (BG)

1 litre

Bordet Gengou agar base (Difco)	30 g
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Glycerol	10 ml
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Suspend 30 g of the powder in 1 L of distilled water containing 10 ml glycerol and mix thoroughly. Heat with frequent agitation and boil for 1 min to completely dissolve the powder, and then autoclave at 121°C for 15 min. Aseptically add 12% sterile, defibrinated blood to the medium at 45-50°C and mix well.

AI.1.3 Cyclodextrin Liquid (CL) medium

1 litre

Sodium-L-glutamate	10.7 g
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L-proline	0.24 g
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Sodium chloride	2.5 g
Potassium di-hydrogen orthophosphate	0.5 g
Potassium chloride	0.2 g
Magnesium chloride (6H ₂ O)	0.1 g
Calcium chloride	0.02 g
Tris	6.1 g
Casamino acids (DIFCO)	10 g
Methyl- β -cyclodextrin	0.25 g

Dissolve the above ingredients in distilled water and adjust pH to 7.6 with HCl, make up to vol., then autoclave at 15 p.s.i. for 15 min. Finally, add vitamin supplement solution to 0.5% (5 ml/L).

Al.1.4 Vitamin Supplement (Solution)

L-cysteine	0.04 g
Ferrous sulphate (7H ₂ O)	0.01 g
Nicotinic acid	0.004 g
Glutathione	0.15 g
Ascorbic acid	0.4 g

Immediately before use, dissolve the ingredients above in 75 ml distilled water, sterilize by membrane filtration.

Al.1.5 Stainer and Scholte (SS-X medium)

1 litre	
L-glutamate (monosodium salt)	10.72 g
L-proline	0.24 g
Sodium chloride	2.5 g

Potassium di-hydrogen orthophosphate	0.5 g
Potassium chloride	0.2 g
Magnesium chloride (6H ₂ O)	0.1 g
Calcium chloride	0.02 g
Tris	6.1 g

Dissolve above ingredients in distilled water (approx. 800ml). Adjust the pH to 7.6 with HCl and make up the volume to 990ml with distilled water. Sterilize the medium by autoclaving at 15 p.s.i. for 15min. Immediately before use, dissolve the ingredients below in 10 ml distilled water, sterilize by membrane filtration and add to the above media.

L-cysteine	0.04 g
Ferrous sulphate (7H ₂ O)	0.01 g
Ascorbic acid	0.02 g
Nicotinic acid	0.004 g
Glutathione	0.1 g

* Cultures with Bvg⁻ (C-mode) characteristics can be produced simply by omitting the 2.5g NaCl from the above medium and adding 5g MgSO₄.7H₂O to give SS-C medium.

AI.1.6 Modified Stainer and Scholte (SS) medium

1 litre

L-glutamate (monosodium salt)	11.84 g
L-proline	0.24 g
Sodium chloride	2.5 g
Potassium di-hydrogen orthophosphate	0.5 g
Potassium chloride	0.2 g
Magnesium chloride (6H ₂ O)	0.1 g
Calcium chloride	0.02 g

Tris	1.5 g
Casamino acids (Becton, Dickinson and Company)	10 g
Methyl- β -cyclodextrin	1 g

Adjust pH to 7.6 and autoclave it. Then, add 5ml of solution below made in 10ml distilled water (after filtration) into the above media.

L-cysteine	0.04 g
Ferrous sulphate (7H ₂ O)	0.01 g
Ascorbic acid	0.4 g
Nicotinic acid	0.004 g
Glutathione	0.15 g

AI.1.7 Casamino acids (CAA) solution

1 litre

Casamino acids (DIFCO)	10 g
Magnesium chloride (6H ₂ O)	0.1 g
Sodium chloride	5 g
Calcium chloride	0.016 g

pH to 7.1;

Autoclave then supplement with 20% sterile (v/v) glycerol for use for storage of *Bordetella* strains at -80°C

AI.1.8 SOC Medium

1 litre

Tryptone (Duchefa Biochemie)	20 g
Yeast extract (Duchefa Biochemie)	5 g
SaltI (250 mM KCl, 1M NaCl)	10 ml

After autoclaving at 15 p.s.i, add 10 ml of 2M sterile glucose and 10 ml of SaltII (1M $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 1M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$)

AI.1.9 Phosphate-buffered saline (PBS)

Sodium chloride	128 mM
Potassium chloride	2.7 mM
Potassium di-hydrogen orthophosphate	1.5 mM
di-potassium hydrogen phosphate	5.0 mM

AI.2 Molecular biology solutions**AI.2.1 Tris-Borate-EDTA (TBE) buffer. 5x stock solution**

1 litre	
Tris-base	54 g
Boric acid	27.5 g
EDTA (0.5 M stock) pH 8.0	20 ml

AI.2.2 DNA loading buffer. 6x stock solution

Tris	60 mM
EDTA	6 mM
Sucrose	40%
Bromophenol blue	0.25%

AI.2.3 Buffer EB

Tris-HCl, pH 8.5	10 mM
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AI.2.4 Buffer TE

pH 8.0

Tris-HCl 10 mM

EDTA 1 mM

AI.3 Protein analysis solutions**AI.3.1 Solutions for SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis)****AI.3.1.1 Protein sample buffer**

Glycerol 5 ml

20% SDS 2.5 ml

2-mercaptoethanol 0.5 ml

Tris (0.5ml, pH 6.8) 2.5 ml

Bromophenol blue 0.25 %

AI.3.1.2 Separating gel

	15 %	12.5 %	10 %	7.5 %
Acrylamide/Bis 30%	5 ml	4.16 ml	3.33 ml	2.5 ml
Tris 1M pH: 8.8	3.75 ml	3.75 ml	3.75 ml	3.75 ml
Distilled water	1.3 ml	2.13 ml	2.97 ml	3.8 ml
SDS 10%	100 µl	100 µl	100 µl	100 µl

APS (Ammonium persulphate)	100 µl	100 µl	100 µl	100 µl
TEMED	10 µl	10 µl	10 µl	10 µl

AI.3.1.3 Stacking gel

30% Acrylamide/Bis (Bio-Rad) 9 ml

Tris 1M pH 6.8 7.5 ml

Distilled water 42 ml

SDS 10% 50 µl

It should be kept at 4°C for further use. Before use, add

APS (Ammonium persulfate) 60 µl

TEMED 5 µl

to the 2 ml of above stacking gel preparation to make 2 gels.

AI.3.1.4 Coomassie gel stain

Coomassie blue 0.5 g

Methanol 400 ml

Acetic acid 100 ml

Distilled water 500 ml

For destain preparation, coomassie blue was omitted from the coomassie gel stain recipe.

AI.3.1.5 PAGE running buffer/Electrode buffer 10X

1 litre

Tris	30 g
Glycine	144 g
SDS	10 g
pH 8.3	

AI.3.1.6 Envelope buffer

Sodium di-hydrogen orthophosphate (NaH_2PO_4)	20 mM
di-sodium hydrogen orthophosphate (Na_2HPO_4)	20 mM

AI.3.2 Western blotting solutions

AI.3.2.1 Electroblotting buffer

Tris base	7.2 g
Glycine	33.4 g

Dissolve the above in 2L distilled water, add 600 ml methanol and make up to 3L with distilled water.

AI.3.2.2 Ponceau S solution

Ponceau S	0.5 g
Glacial acetic acid	1 ml

Make up to 100 ml with distilled water.

AI.3.2.3 Blocking buffer

Blotto (Marvel skimmed milk)	6 g
Tween-20	400 μl
PBS	200 ml

AI.3.2.4 Washing buffer

Tween-20	400 μl
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PBS	200 ml
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AI.3.2.5 DAB solution

3,3'-Diaminobenzidine (DAB)	0.05 g
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PBS	98 ml
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Filter through Whatman paper 1MM

1% CoCl ₂	2 ml
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30% H ₂ O ₂	100 µl
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AI.4 Southern blot solutions and buffers

AI.4.1 Hybridization solutions

AI.4.1.2 Depurination solution

Hydrochloric acid (HCl)	0.25 M
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AI.4.1.3 Denaturation solution

Sodium hydroxide	0.5 M
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Sodium chloride	1.5 M
-----------------	-------

AI.4.1.4 Neutralization solution

Sodium chloride	3 M
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Tris-HCl	0.5 M, pH 7.5
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AI.4.1.5 N-lauroylsarcosine stock solution

10% N-lauroylsarcosine (w/v) in sterile H₂O

Filtered through a 0.2-0.45 µm membrane

AI.4.1.6 SDS stock solution

10% SDS (w/v) in sterile H₂O

Filtered through a 0.2-0.45 µm membrane

AI.4.1.7 Standard prehybridization buffer

5x SSC	88.8 ml
N-lauroylsarcosine stock solution	1 ml
SDS stock solution	200 µl
Blocking reagent stock solution	10 ml

AI.4.2 Detection solutions and buffers

AI.4.2.1 Maleic acid buffer

Sodium chloride	0.15 M
Maleic acid	0.1 M

Adjust pH to 7.5 at 20°C with concentrated or solid NaOH; autoclave.

AI.4.2.2 Washing buffer / Equilibrating solution

Maleic acid	0.1 M
Sodium chloride	0.15 M
Tween-20	0.3 %

0.3% (v/v) Tween-20 was added to sterile Maleic acid buffer. (it should not be autoclaved while containing Tween-20).

AI.4.2.3 Blocking reagent stock solution

Blocking Reagent (Boehringer Mannheim) is dissolved in maleic acid buffer to a final concentration of 10% (w/v) with shaking and heating at 60°C for 1 h or until completely in solution. The solution was autoclaved using a regular program, such as that used for the sterilization of cell culture medium. Blocking reagent must be completely in solution before autoclaving. Autoclaved solution was stored at +4°C and checked before each use for contamination.

AI.4.2.4 Blocking buffer / Antibody blocking solution

Blocking reagent stock solution (10%)	20 ml
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Maleic acid buffer 180 ml

Diluted Blocking reagent stock solution 1:10 with Maleic acid buffer (1% final blocking reagent concentration). Dissolved with heating and shaking. The anti-Digoxigenin-AP antibody was added at 1:10000 dilution in blocking buffer.

AI.4.2.5 Detection buffer

Tris-HCl 0.1 M, pH 9.5 (at 20°C)

Sodium chloride 0.1 M

AI.4.2.6 TE buffer

Tris-HCl 10 mM

EDTA 1 mM

pH 8.0 (at 20°C)

AI.4.2.7 Colour substrate solution

(Freshly prepared)

45 µl NBT (Nitro-Blue Tetrazolium Chloride) solution and 35 µl BCIP (5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt) solution were added to 10 ml Detection buffer.

AI.4.3 General solutions

20 x SSC solution

Sodium chloride 3 M

Sodium citrate 0.3 M

pH 7.0 (at 20°C), autoclaved at 15 psi for 15 min

Washing solution 2x

(for low stringency washing)

2x SSC

0.1 % SDS

Washing solution 0.5x

(for high stringency washing)

0.5 x SSC

0.1% SDS

Stripping solutions

Dimethylformamide dimethylformamide (DMF)	100% ACS (American Chemical Society) grade N,N-
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H ₂ O	Sterile, distilled water
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Alkaline probe-stripping solution	0.2 NaOH, 0.1% SDS
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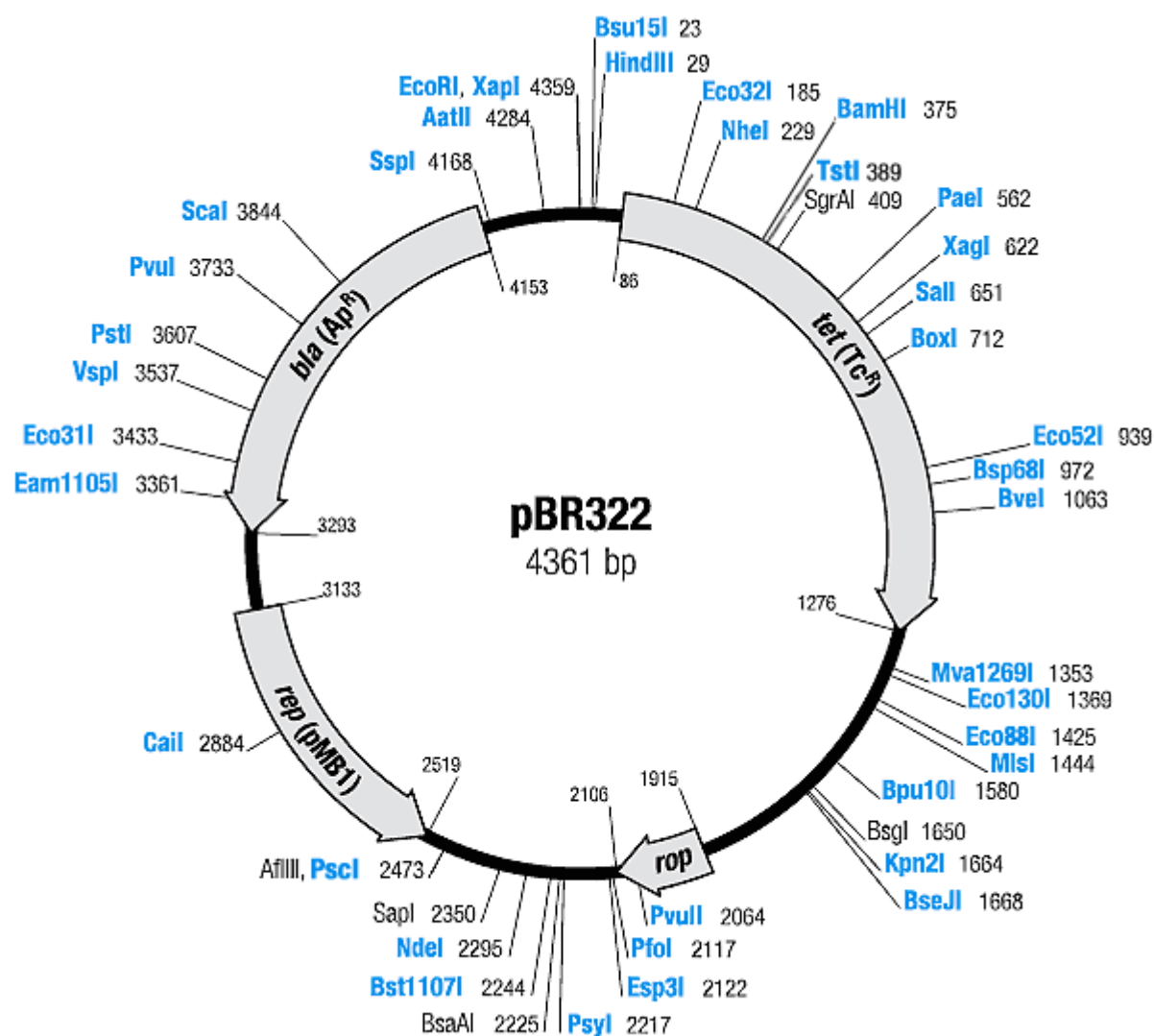
2×SSC buffer	300 mM NaCl, 30 mM sodium citrate
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2×SSC / 0.1% SDS	300 mM NaCl, 30mM sodium citrate, 0.1%(w/v) SDS
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6.2 Appendix II

All.1 Map of the plasmid pBR322

(<http://www.fermentas.com/techinfo/nucleicacids/mappbr322.htm>)



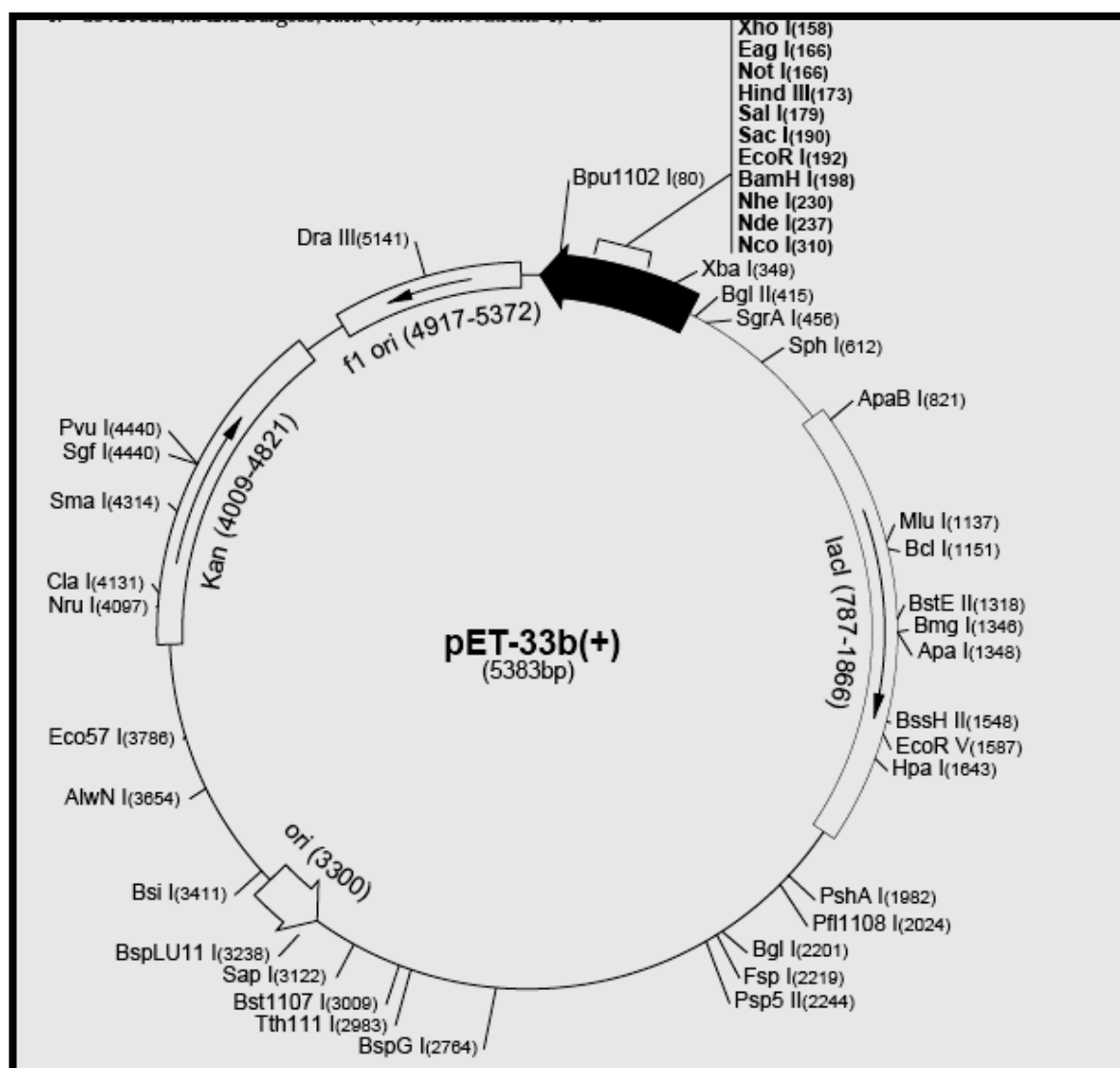
The plasmid pBR322 is 4361 bp in length and contains:

(1) the replicon *rep* responsible for the replication of plasmid (source - plasmid pMB1); (2) *rop* gene coding for the Rop protein, which promotes conversion of the unstable RNA I - RNA II complex to a stable complex and serves to decrease copy number (source - plasmid pMB1); (3) *bla* gene, coding for beta-lactamase that confers resistance to ampicillin (source - transposon Tn3); (4) *tet* gene, encoding tetracycline resistance protein (source - plasmid pSC101)(<http://www.fermentas.com/techinfo/nucleicacids/mappbr322.htm>). The

circular sequence is numbered such that 1 is the first T of the unique EcoRI site GAATTC and the count increases first through the *tet* gene, the pMB1 material, and finally through the Tn3 region. The map shows enzymes that cut pBR322 DNA once. The coordinates refer to the position of first nucleotide in each recognition sequence.

All.2 Map of pET-33b (+) expression vector

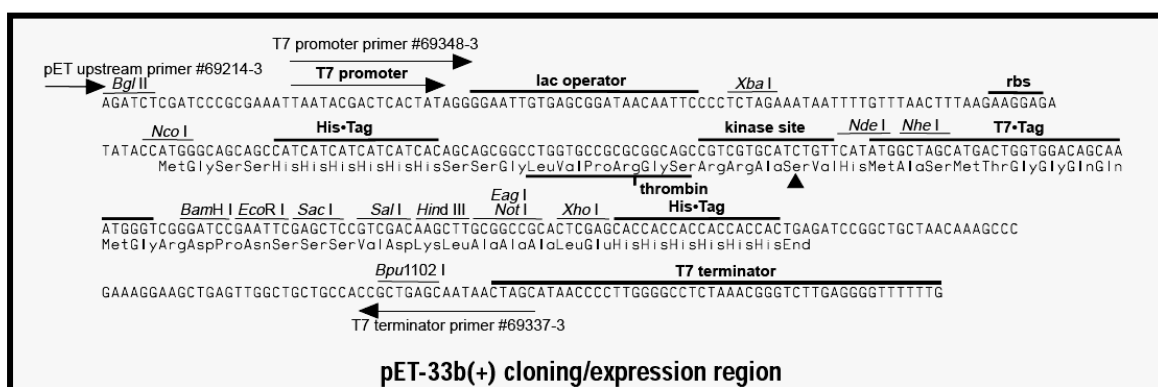
(<http://www.merckbiosciences.com/docs/docs/PROT/TB147.pdf>)



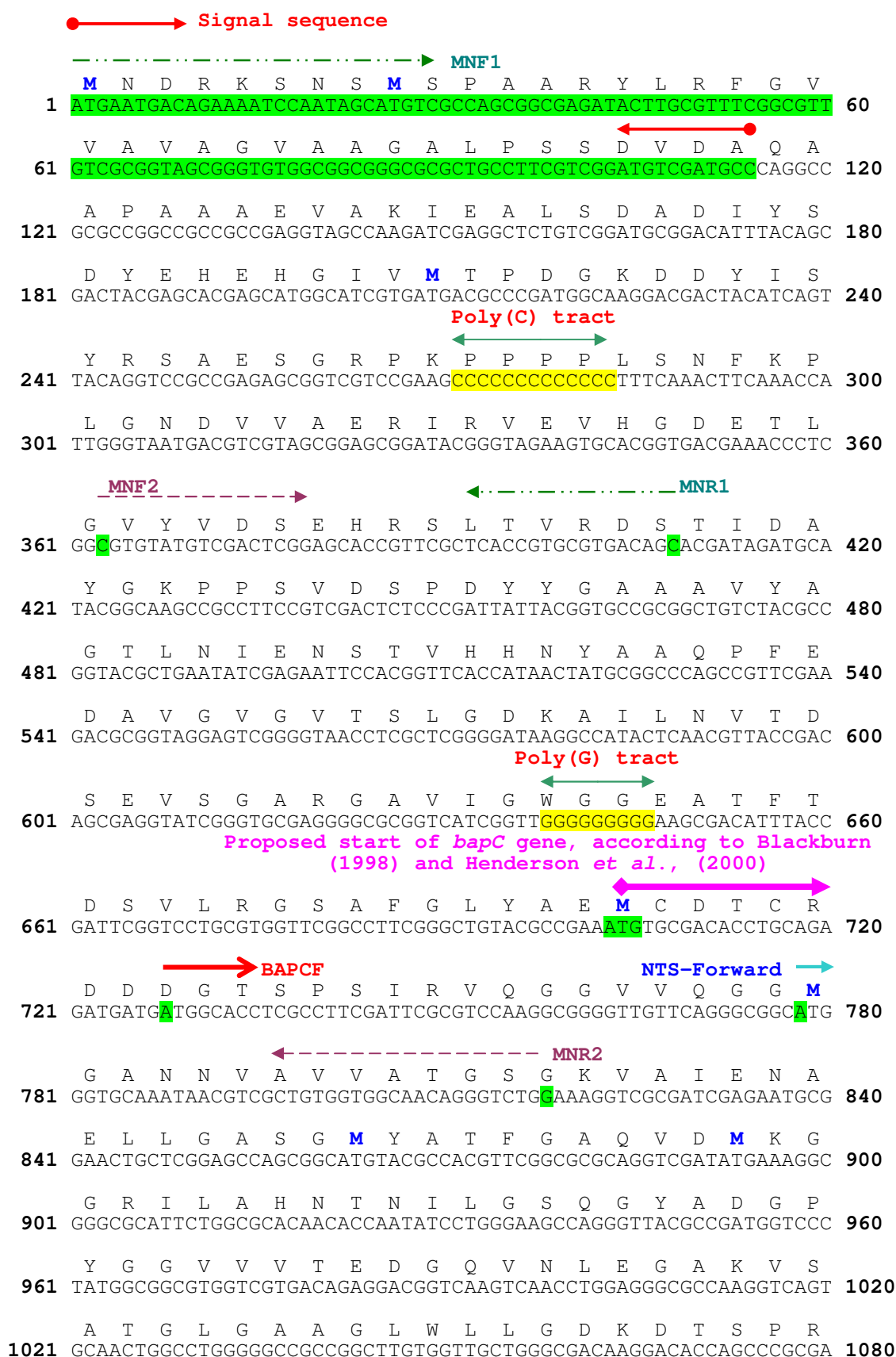
pET-33b(+) sequence landmarks

T7 promoter	384-400		
T7 transcription start	383	His•Tag coding sequence	140-157
His•Tag coding sequence	284-301	T7 terminator	26-72
PKA site coding sequence	242-256	<i>lacI</i> coding sequence	787-1866
T7•Tag® coding sequence	206-238	pBR322 origin	3300
Multiple cloning sites (<i>BamH</i> I - <i>Xho</i> I)	158-203	Kan coding sequence	4009-4821

The pET-33b(+) vector (Novagen, Cat. No. 69054-3) is derived from pET-28b(+) and carries a 15bp sequence encoding the protein kinase A (PKA) site RRASV, located between the thrombin cleavage and *Nde* I sites. Unique sites are shown on the above circle map. Note that the sequence is numbered by the pBR322 convention, so the T7 expression region is reversed on the circle map.



All.3 Significant features of *bapC* gene and BapC protein



A S L R N T D V H G E V A A I A L G F N
 1081 GCCAGCCTGCGCAACACCGACGTCCACGGAGAGGTCGCCGCCATTGCGCTGGGGTTCAAT 1140
 G E A N I S G G S L S V E D G A V L T T
 1141 GGCAGGGCGAACATCTCGGGCGGCAGCTTGAGCGTAGAGGATGGGGCCGTGCTCACCACC 1200
 Tetracycline cassette insertion (*Nco*I)
 NTS-Reverse (1244)
 L T P D A V E Y Y Y D Y A L S M E H L P
 1201 CTGACGCCCCGATGCAGTCGAGTATTACTACGA TACGCTTGT CATGG AGCATCTGCCA 1260
 A D A P L T P V R V T L S D G A R A S G
 1261 GCTGATGCGCCGTTGACGCCGGTCCGCGTCACGCTGTCCGATGGCGCGCGGCCAGCGGA 1320
 E T L I A H G G L L P M T L R L S S G V
 1321 GAAACGTTGATCGCGCATGGCGGGTTGTTGCCCATGACGCTGCGCTTGAGCAGCGGGGTC 1380
 RGD motif
 D A R G D I V T L P P S A P P D S A E Q
 1381 GACGCC CGCGGCGAC ATCGTCACGCTGCCGCCTTCCGCGCCGCCGATTCCGCGGAGCAA 1440
 P D A E P E P D A E L E P D A A A Q S D
 1441 CCGGATGCCGAGCCGGAACCGGATGCCGAGCTGGAACCGACGCCGCGGCGCAGTCGGAC 1500
 A K A N A R V M A Q V D G G E P V A V P
 1501 GCCAAGGCGAATGCGCGGGTCATGGCGCAGGTAGATGGCGGGGAACCTGTTGCCGTGCCG 1560
 I P A P S H P D A P I D V F I D S G A Q
 1561 ATCCCGGCCCCCTTCGCATCCCGATGCCCCGATCGACGTGTTTCATCGACAGCGGTGCCAA 1620
 W R G M T K T V N A L R I E D G T W T V
 1621 TGGCGGGGCATGACCAAGACCGTCAATGCGTTGCGCATCGAGGACGGCACCTGGACCGTC 1680
 T G S S T V N S L H L Q A G K V A Y A T
 1681 ACCGGGTCGTCCACGGTGAACAGCCTGCACCTGCAGGCAGGCAAGGTGGCGTACGCAACG 1740
 Glycosaminoglycan-binding motif
 P A E S D G E F K H L R V K T L S G S G
 1741 CCTGCCGAAAGCGACGGAGAATTCAAACACCTGCGGGTCAAGACCCTC TCGGGAAGCGGC 1800
 L F E M N A S A D L S D G D L L V V S D
 1801 CTGTTTCGAGATGAACGCCAGCGCCGACCTGAGCGATGGCGACCTGCTGGTCGTGTCCGAC 1860
 E A S G Q H K V L V R G A G T E P T G V
 1861 GAGGCCAGCGGGCAGCACAAGGTGCTGGTGGCAGGAGCCGGCACGGAACCCACCGGTGTG 1920
 E S L T L V E L P E G S Q T K F T L A N
 1921 GAAAGCCTGACGCTGGTTCGAGCTGCCCCGAGGGCAGCCAGACGAAGTTCACGCTTGCCAAC 1980
 R G G V V D A G A F R Y R L T P D N G V
 1981 CGGGGCGGGGTGGTTCGACGCCGGCGCGTTCCGCTATCGCCTGACGCCGACAAACGGTGTG 2040
 W G L E R T S Q L S A V A N A A L N T G
 2041 TGGGGCCTGGAACGGACCGACCGAGCTTTTCGGCCGTCGCCAACGCGGCCTTGAATACCGG 2100
 Putative cleavage site
 G V G A A S S I W Y A E G N A L S K R L
 2101 GCGTGGGCGCGGCCAGCAGCATCTGGTATGCGGAAGGCAAT GCGCTCTCCAAGCGCCTG 2160
 G E L R L D P G A G G F W G R T F A Q K
 2161 GCGAGTTG CGGCTCGATCCCGGCGCGGGCGGCTTCTGGGGGCGCACGTTTCGCCAGAA 2220
 Q Q L D N K A G R R F D Q K V Y G F E L
 2221 CAGCAGCTCGACAACAAGGCTGGCCGACGCTTCGACCAGAAGGTGTACGGTTTCGAGCTG 2280
 G A D H A I A G Q Q G R W H V G G L L G
 2281 GGGGCCGACCATGCCATCGCAGGACAGCAAGGGCGCTGGCACGTGGGCGGCCTGCTGGGC 2340

Y T R A R R S F I D D G A G H T D S A H
 2341 TATACCCGCGCAAGGCGCAGCTTCATCGATGACGGCGCCGGGCATACCGACAGCGCGCAT 2400

 I G A Y A A Y V A D N G F Y F D S T L R
 2401 ATCGGGGCTACGCGGCGTACGTGGCGGACAACGGCTTCTATTTTCGATTCGACCCCTGCGC 2460

 A S R F E N D F T V T A T D A V S V R G
 2461 GCCAGCCGCTTCGAGAACGACTTCACGGTAACGGCCACCGACGCCGTTTCCGTACGGGGC 2520

 K Y R A N G V G A T L E A G K R F T L H
 2521 AAGTACCGGGCCAATGGGGTAGGCGCCACCTTGGAGGCCGGCAAACGTTTCACGTTGCAC 2580

 D G W F V E P Q S E V S L F H A S G G T
 2581 GACGGCTGGTTCGTCTGAACCTCAGTCCGAGGTGTCGCTGTTCCATGCCAGCGGCGGAACC 2640

 Y R A A N N L S V K D E G G T S A V L R
 2641 TACCGTGCCGCGAACAACCTGTCCGTCAAGGACGAAGGCGGCACCTCCGCCGTGCTGCGC 2700

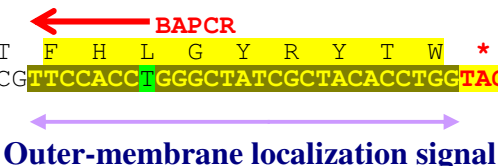
 L G L A A G R R I D L G K D R V I Q P Y
 2701 CTGGGCTTGGCGGCCGGGCGACGCATCGACCTGGGCAAGGACCGCGTGATCCAGCCCTAT 2760

 A T L S W L Q E F K G V T T V R T N G Y
 2761 GCCACCCTGAGCTGGCTGCAGGAATTCAAAGGCGTCACGACCGTTCGCACCAACGGGTAC 2820

 G L R T D L S G G R A E L A L G L A A A
 2821 GGGCTGCGCACCGACCTGAGCGGTGGCCGGGCTGAATTGGCGCTGGGCTGGCCGCCGCG 2880

 L G R G H Q L Y T S Y E Y A K G N K L T
 2881 TTGGGGCGCGGCCACCAGCTCTACACTTCGTACGAGTACGCCAAGGGCAACAAGCTGACC 2940

 L P W T F H L G Y R Y T W *
 2941 TTGCCTTGGACGTTCCACCTGGGCTATCGCTACACCTGGTAG 2982



Outer-membrane localization signal

The gene/protein sequence was obtained from the NCBI and shows the full length sequence without the frameshifts known to be present in the BP Tohama genome sequence. The first green highlighted segment is the signal sequence with 38 amino acids. The poly(C) and poly(G) tracts are highlighted in yellow with primer pairs, MNF1/MNR1 and MNF2/ MNR2, used to amplify these fragments, respectively. Other features of BapC such as RGD motif, glycosaminoglycan-binding site (SGXG), putative cleavage site and outer-membrane localization signal are highlighted.

All.4 *brkA* gene mutated by transposon 5 insertion and primers (Fernandez and Weiss, 1994)

ATGTATCTCGATAGATTCCGTCAATGTCCGTCTTCTTGACAGATCCCGCGTTCCGCGTGGCGCCTGCATG
 CGCTGGCCCGCAGCTCTGGCGCTGGCCGGCATGGCCCGCTGGCGCCCGCGGCGCGCAGGCGCCGAGCC
 GCCCGTGGCCGGCGCGCCGCATGCGCAGGACGCCGGGCAGGAAGGAGAGTTTCGACCACCGGGACAACACG
 CTCATTGCAGTCTTTGACGACGGCGTCGGCATCAATCTCGACGACGATCCCGACGAGCTCGGCGAGACGG
 CGCCCCCACGCTCAAGGACATCCACATCTCGGTGGAGCACAAGAACCCGATGAGCAAGCCGGCCATCGG
 GGTGCGTGTGACGCGCGCCGGCCGCGCGCTGACGCTGGCCGGCTCGACCATCGATGCCACCGAGGGCGGC
 ATTCGCGCAGTGGTACGGCGCGGCGGCACGCTGGAGCTGGATGGCGTACCCGTGGCGGGCGGGGAAGGGA
 TGGAGCCGATGACGGTCTCTGACGCCGGCAGCCGCTGAGCGTGCAGCGGCGGCGTGTGGGCGGCGAAGC
 GCCGGGCGTTCGGCCTGGTCCGGGCGCGCAAGGCGGCCAGGCGAGCATCATCGACGCGACGCTGCAGAGC
 ATCCTCGGGCCCGCGCTCATTGCCGACGGCGGCTCCATTTCCGTGCGCGGCGGTTTCGATCGACATGGACA
 TGGGCCCCGGGATTCCCGCCCGCCCTCCACCGCTTCCCGGGGCGCGCTGGCCGCGCATCCGCCGCTCGA
 TCGCGTTGCCGCGGTGCACGCCGGCCAGGACGGCAAGGTGACACTGCGGGAGGTGGCGCTGCGGGCTCAC
 GGGCCGAGGCGACGGGCGCTCTATGCGTATATGCTTGGCAGCGAAATCACCTGCGAGGAGGCACAGTCA
 GCGTGCAGGGCGATGACGGGGCCGGCGTGGTTCGCCGGCGCGGGCCTGCTCGACGCGCTTGCCGCCCGGCGG
 CACGGTGCAGGCTGGACGGAACACGGTGTGACCGATGGCGCCAACACCGATGCCGTGCTGGTTTCGCGGC
 GACGCGGCGCGCGCCGAGGTGCTCAACACCGTGCTGCGCACCGCCAAGAGCCTGGCCGCCGGCGTATCGG
 CCCAGCATGGAGGCCGCGTACGCTGCGGCAGACCCGCATCGAGACCGCGGGCGCGGGGGCCGAGGGCAT
 CTCCGTGCTGGGCTTCGAGCCGAGTCCGGCTCCGGCCCGGCCAGCGTCGACATGCAGGGCGGCAGCATC
 ACCACGACCGGCAACCGCGCCCGGCATCGCGCTACCCACGGCAGCGCCCGCTGGAAGGCGTGGCGG
 TGCAGCGCGAGGGCAGCGGCTCGAGCGCCGCGCAGCTGGCCAACGGCACGCTGGTCTGTCAGCGCAGGGTC
 GCTGGCCTCGGCCCAGTCCGGCGCGATCAGCGTGACCGACACGCCGCTGAAGTGTATGCCGGGCGCCCTG

 BRKF →
 GCCAGCAGCACGGTCTCGGTGCGGGTTGACCGACGGCGCCACGGCGCAAGGCGGCAATGGCGTGTTCCTCC
 AGCAGCATTCACCATTCGGTGGCGGTTGCCCTCGAGAGCGGCGCCCTGGCTCGCGGCGATATCGTCGC
 CGACGGCAACAAGCCCTCGATGCCGGGATCTCCCTCAGCGTGGCCAGCGGCGCCGCTGGCACGGCGCC
 ACCCAGGTGCTCCAGTCCGCCACGCTGGGCAAGGGCGGAACCTGGGTCTGTAACGCCGACTCCCGGGTGC
 AGGACATGTGATGCGCGGCGGGCGGGTGAATTCCAGGCGCCCGCGCCGAGGCCTCTTACAAGACCCCT
 GACCCTGCAAACCTGGACGGCAACGGCGTGTTCGTGCTGAACACCAACGTCGCCGCCGGGCGAGAACGAC
 CAGTTGCGGGTACCGGCCGCGCCGATGGCCAGCACCGCGTGTGTTGCGCAATGCCCGAGGCGAGGCCG


 ACAGCCGGGGCGCCCGCTGGGCTGGTGCATACCCAGGGGCGAGGGCAACGCCACCTTCCGGCTGGCCAA
 CGTCGGCAAGGCGGTTGACCTGGGCACGTGGCGCTACAGCCTGGCGGAGGATCCGAAGACGCATGTCTGG
 AGCTTGACGCGCGCGGGCCAGGCCCTGTGCGGGGCGGCCAATGCCCGCTGAACGCGGCGGATCTTTCCA
 GCATCGCCCTGGCCGAGTCCAACGCGCTGGACAAGCGCTGGGCGAGCTGCGCCTGCGCGCCGACGCCGG
 CGGGCCATGGGCGCGTACGTTACGCGAGCGCCAGCAGATCAGCAACCGCCACGCCCGCGCCTACGACCAG
 ACGGTACGCGGGCTGGAGATCGGCCTGGACCGTGGCTGGAGCGCGTGGGCGGGCGCTGGTACGCCGGCG
 GCCTGCTCGGCTACACCTATGCCGACCGCACCTATCCCGCGCAGGTGGCGGCAAGGTCAAGGGCCTGCA
 CGTCGGCGGCTACGCCGCTATGTGCGCGATGGCGGCTACTATCTCGACACCGTGTGCGGCTGGGCCGC
 TACGATCAGCAATACAACATTGCCGGCACCGATGGCGGCCGCGTCACCGCCGACTACCGCACAAAGCGGCG
 CCGCATGGTCTGCTCGAAGGCGGGCGCCGGTTCGAGCTGCCCCAACGACTGGTTTCGCCGAACCGCAGGCCGA
 GGTCATGCTGTGGCGCACGTACGGCAAGCGCTATCGCGCCAGCAATGGCCTGCGCGTCAAGGTGGACGCC
 AACACCGCCACGCTGGGCCGCTGGGCTTGCCTTCGGCCGCCGCATCGCCCTGGCCGGCGGCAACATCG
 TGCAGCCCTACGCCAGGCTCGGCTGGACGCGAGGAGTTCAAAAGCACGGGCGATGTGCGCACCAATGGCAT
 TGGCCATGCCGGCGCAGGCCGCCACGGCCGCGTGGAACTGGGCGCGGGCGTTCGACGCCGCTTGGGCAAG
 GGGCACAACCTCTATGCTTCGTACGAGTACGCGGCGGGCGACCGGATCAACATTCCGTGGTCTGTTCCACG
 CCGGCTACCGCTACAGTTCTGA
 ← BRKR2

brkA gene forward primer (BRKF) nucleotide

brkA gene reverse primer (BRKR2) nucleotide

↓ *Tn5* transposon inserted in position 3232 in *brkA* gene

All.5 BLAST2 sequence comparison of SapA protein of *S. typhimurium* with BapC of *B. pertussis*

 Blast 2 Sequences results

[PubMed](#) [Entrez](#) [BLAST](#) [OMIM](#) [Taxonomy](#) [Structure](#)

BLAST 2 SEQUENCES RESULTS VERSION BLASTP 2.2.15 [Oct-15-2006]

[Matrix](#) gap open: gap extension:

x_dropoff: expect: wordsize: [Filter](#) ☒ View option

Masking character option

Masking color option

☐ Show CDS translation

Sequence 1: lcl|SapA *Salmonella typhimurium*
Length = 549

Sequence 2: lcl|BapC *Bordetella pertussis*
Length = 993

No significant similarity was found


CPU time: 0.02 user secs. 0.00 sys. secs 0.02 total secs.

Lambda K H
0.319 0.133 0.402

Gapped
Lambda K H
0.267 0.0410 0.140

Matrix: BLOSUM62
Gap Penalties: Existence: 11, Extension: 1
Number of Sequences: 1
Number of Hits to DB: 3064
Number of extensions: 2093
Number of sequences better than 10.0: 0
Number of HSP's gapped: 0
Number of HSP's successfully gapped: 0
Length of query: 549
Length of database: 1,512,095,185
Length adjustment: 140
Effective length of query: 409
Effective length of database: 1,512,095,045
Effective search space: 618446873405
Effective search space used: 618446873405
Neighboring words threshold: 9
X1: 16 (7.4 bits) X2: 129 (49.7 bits) X3: 129 (49.7 bits)
S1: 41 (21.8 bits) S2: 82 (36.2 bits)

All.6 BLAST2 sequence comparison of SapA protein of *S. typhimurium* with BrkA of *B. pertussis*

 Blast 2 Sequences results

[PubMed](#) [Entrez](#) [BLAST](#) [OMIM](#) [Taxonomy](#) [Structure](#)

BLAST 2 SEQUENCES RESULTS VERSION BLASTP 2.2.15 [Oct-15-2006]

[Matrix](#) gap open: gap extension:

x_dropoff: expect: wordsize: [Filter](#) ☒ View option

Masking character option

Masking color option

☐ Show CDS translation

Sequence 1: lcl|**SapA** *Salmonella typhimurium*
Length = 549

Sequence 2: lcl|**BrkA** *Bordetella pertussis*
Length = 1010

No significant similarity was found

CPU time: 0.02 user secs. 0.00 sys. secs 0.02 total secs.

Lambda	K	H
0.319	0.133	0.402

Gapped

Lambda	K	H
0.267	0.0410	0.140

Matrix: BLOSUM62

Gap Penalties: Existence: 11, Extension: 1

Number of Sequences: 1

Number of Hits to DB: 3150

Number of extensions: 2209

Number of sequences better than 10.0: 0

Number of HSP's gapped: 0

Number of HSP's successfully gapped: 0

Length of query: 549

Length of database: 1,512,170,332

Length adjustment: 140

Effective length of query: 409

Effective length of database: 1,512,170,192

Effective search space: 618477608528

Effective search space used: 618477608528

Neighboring words threshold: 9

X1: 16 (7.4 bits) X2: 129 (49.7 bits) X3: 129 (49.7 bits)

S1: 41 (21.8 bits) S2: 82 (36.2 bits)

All.7a Mascot Search Results of full-length BapC protein expressed in *E. coli*



User : rb
 Search title : Sample1
 MS data file : C:\Documents and Settings\rjb12n\My Documents\Mojtaba.wiff
 Database : NCBIInr 20070524 (4970641 sequences; 1719627930 residues)
 Taxonomy : Bacteria (Eubacteria) (2395850 sequences)

Protein hits :	gi 33593700	putative autotransporter (pseudogene) [Bordetella pertussis Tohama I]
	gi 33597172	putative autotransporter [Bordetella parapertussis 12822]
	gi 15803853	elongation factor EF-2 [Escherichia coli O157:H7 EDL933]
	gi 15799798	pyruvate dehydrogenase (decarboxylase component) [Escherichia coli O157:H7 EDL933]
	gi 24111558	pyruvate dehydrogenase (decarboxylase component) [Shigella flexneri 2a str. 301]
	gi 147365	ATP-dependent protease binding subunit [Escherichia coli]
	gi 56480150	heat shock protein [Shigella flexneri 2a str. 301]
	gi 16759149	pyruvate dehydrogenase E1 component [Salmonella enterica subsp. enterica serovar Typhi str. CT18]
	gi 15799694	molecular chaperone DnaK [Escherichia coli O157:H7 EDL933]
	gi 16123568	pyruvate dehydrogenase E1 component [Yersinia pestis C092]
	gi 15799802	aconitate hydratase [Escherichia coli O157:H7 EDL933]
	gi 16759005	molecular chaperone DnaK [Salmonella enterica subsp. enterica serovar Typhi str. CT18]
	gi 43019	unnamed protein product [Escherichia coli]
	gi 15800430	2-oxoglutarate dehydrogenase (decarboxylase component) [Escherichia coli O157:H7 EDL933]
	gi 146312713	ATPase AAA-2 domain protein [Enterobacter sp. 638]
	gi 37524440	translation elongation factor EF-G [Phototribadus luminescens subsp. laumondii TT01]
	gi 21672436	DnaK protein [Buchnera aphidicola str. Sg (Schizaphis graminum)]
	gi 114773099	ATP-binding subunit of Clp protease and DnaK/DnaJ chaperones [alpha proteobacterium HTCC2255]
	gi 146642	protease La (lon)
	gi 56460289	ATP-binding subunit of Clp protease and DnaK/DnaJ chaperones [Idiomarina loihiensis L2TR]
	gi 123444103	elongation factor G [Yersinia enterocolitica subsp. enterocolitica 8081]

All.7b Mascot Search Results of full-length BapC protein expressed in *E. coli*

The protein was expressed by *E. coli* BL21 (DE3) pLysS containing plasmid pET33b (BapC + SS).

Match to: **gil33593700** Score: **1576**

autotransporter (pseudogene) [Bordetella pertussis Tohama I] (locus_tag BP2738)

Nominal mass (M_r): **104098**; Calculated pI value: **4.98**

NCBI BLAST search of [gil33593700](#) against nr

Sequence Coverage: **46%**

Matched peptides shown in **Bold Red**

1	MNDRKSNSMS	PAARYLRFV	VAVAGVAAGA	LPSSDVDAQA	APAAAEVAKI
51	EALSDADIYS	DYEHEHGIVM	TPDGKDDYIS	YRSAESGRPK	PPPPLSNFKP
101	LGNDVVAERI	RVEVHGDETL	GVY VDSEHRS	LTVRDSTIDA	YGKPPSVDSP
151	DYYGAAAVYA	GTLNIENSTV	HHNYAAQPFE	DAVGVGVTSL	GDKAILNVTD
201	SEVSGARGAV	IGWGGEATFT	DSVLRGSAFG	LYAEMCDTCR	DDDGTSPSIR
251	VQGGVVQGM	GANNVAVVAT	GSGKVAIENA	ELLGASGMYA	TFGAQVDMKG
301	GRILAHNTNI	LGSQGYADGP	YGGVVVTEDE	QVNLEGAK VS	ATGLGAAGLW
351	LLGDKDTSR	ASLRNTDVHG	EVAALALGFN	GEANISGGSL	SVEDGAVLTT
401	LTPDAVEYYY	DYALSMEHLP	ADAPLTPVRV	TLSDGAR ASG	ETLIAHGGLL
451	PMTLR LSSGV	DARGDIVTLP	PSAPPDSAEQ	PDAEPEPDAE	LEPDAAAQSD
501	AKANARVMAQ	VDGGEPAVP	IPAPSHPDAP	IDVFIDSGAQ	WRGMTKTUNA
551	LR IEDGTWTV	TGSSTVNSLH	LQAGKVAYAT	PAESDGEFKH	LRVKTLSGSG
601	LFEMNASADL	SDGDLVVS	EASGQHKVLV	RGAGTEPTGV	ESLTLVELPE
651	GSQTK FTLAN	GGVVDAGAF	RYRLTPDNGV	WGLERTSQLS	AVANAALNTG
701	GVGAASSIWY	AEGNALSRL	GELRLDPGAG	GFWRGRTFAQK	QQLDNKAGRR
751	FDQK VYGFEL	GADHAIAGQQ	GRWHVGGLLG	YTRARRSFID	DGAGHTDSA
801	IGAYAAAYVAD	NGFYFDSTLR	ASR FENDFTV	TATDAVSVRG	KYRANGVGAT
851	LEAGKR FTLH	DGWFVEPQSE	VSLEFHASGGT	YRAANNLSVK	DEGGTSAVLR
901	LGLAAGRRID	LGK DRVIQPY	ATLSWLQEFK	GVTTVRTNGY	GLR TDLSSGR
951	AELALGLAAA	LGRGHQLYTS	YEYAKGNKLT	LPWTFHLGYR	YTW